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A role for loop G in the β 1 strand in GABA_A receptor activation

Abbreviated title: Loop G in GABA_AR

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Key points summary

- The role of the β 1 strand in GABA_A receptor function is unclear. It lies anti-parallel to the β 2 strand, which is known to participate in receptor activation.
- Molecular dynamics simulation revealed solvent accessible residues within the β 1 strand of the GABA_A β 3 homopentamer that might be amenable to analysis using the substituted Cys accessibility method.
- Cys substitutions from Asp43 to Thr47, in the GABA_A α 1 subunit showed that D43C and T47C reduced apparent potency of GABA. F45C caused a bi-phasic GABA concentration-response relationship and increased spontaneous gating.
- Cys43 and Cys47 were accessible to MTSEA modification, while Cys45 was not. Both GABA and the allosteric agonist propofol reduced MTSEA modification of Cys43 and Cys47.
- By contrast, modification of Cys64 in the β 2 strand loop D was impeded by GABA but unaffected by propofol.
- These data reveal movement of β 1 strand loop G residues during agonist activation of the GABA_A receptor.

Abstract

The GABA_A receptor α subunit β 1 strand runs anti-parallel to the β 2 strand, which contains loop D, known to participate in receptor activation and agonist binding. However, a role for the β 1 strand has yet to be established. We used molecular dynamics simulation to quantify the solvent accessible surface area (SASA) of β 1 strand residues in the GABA_A β 3 homopentamer structure. Residues in the complementary interface equivalent to those between Asp43 and Thr47 in the α 1 subunit have an alternating pattern of high and low SASA consistent with a β strand structure. We investigated the functional role of these β 1 strand residues in the α 1 subunit by individually replacing them with Cys residues. D43C and T47C substitutions reduced the apparent potency of GABA at α 1 β 2 γ 2 receptors by around 50-fold and 8-fold, respectively, whereas the F45C substitution caused a biphasic GABA concentration-response relationship and increased spontaneous gating. Receptors with D43C or T47C substitutions were sensitive to MTSEA modification. However, GABA-evoked currents mediated by α 1(F45C) β 2 γ 2 receptors were unaffected by MTSEA, suggesting that this residue is inaccessible. Both GABA and the allosteric agonist propofol reduced MTSEA modification of α 1(D43C) β 2 γ 2 and α 1(T47C) β 2 γ 2 receptors indicating movement of the β 1 strand even during allosteric activation. This is in contrast to α 1(F64C) β 2 γ 2 receptors where only GABA, but not propofol reduced MTSEA modification. These findings provide the first functional evidence for movement of the β 1 strand during gating of the receptor and identify residues that are critical for maintaining GABA_A receptor function.

Abbreviations list

DMEM, Dulbecco modified Eagle's medium; HEK-293, human embryonic kidney 293 cell; MD, molecular dynamics; MTS, methanethiosulfonate; MTSEA, 2-aminoethyl methanethiosulfonate; pLGIC, pentameric ligand gated ion channel; SASA, solvent accessible surface area; SCAM, substituted cysteine accessibility method; TM, transmembrane; τ_w , weighted tau.

Introduction

γ -Aminobutyric acid type A (GABA_A) receptors are members of the Cys-loop family of pentameric ligand-gated ion channels (pLGICs). GABA_A receptors are assembled from 19 different subunits. The most common synaptic GABA_A receptor is comprised of α 1, β 2 and γ 2 subunits (Whiting et al., 1995).

GABA_A receptors have a large extracellular domain, housing the orthosteric ligand binding site, and four transmembrane (TM) domains (TM1-4), which contain binding sites for allosteric agonists and several non-competitive antagonists. TM2 lines the Cl⁻-selective channel pore. Like most pLGICs GABA_A receptors also have a large intracellular domain, mainly composed of the TM3-4 loop (Baptista-Hon et al., 2013).

The orthosteric binding site, located at the interface between adjacent subunits (Smith & Olsen, 1995; Cromer *et al.*, 2002), is lined by residues within six non-continuous loops (A-F) which participate in binding (Boileau *et al.*, 1999; Holden & Czajkowski, 2002; Wagner *et al.*, 2004; Goldschen-Ohm *et al.*, 2011; Tran *et al.*, 2011) and gating (Boileau *et al.*, 2002; Newell & Czajkowski, 2003; Venkatachalan & Czajkowski, 2008; Szczot *et al.*, 2014). In GABA_A receptors, the primary interface contributes loops A, B and C, from the β subunit, while the complimentary interface contributes loops D, E and F, from the α subunit. These loops are contained within an anti-parallel β sandwich structure, which makes up much of the N-terminal domain.

Several pLGIC structures have recently been solved, some in the presence of agonists providing an insight into the architecture of the orthosteric binding site. A structural model of *C. elegans* GluCl implicates β 1 strand Arg37 in glutamate binding (Hibbs & Gouaux, 2011). This led to the proposal of a seventh binding loop, termed loop G. However, the equivalent β 1 strand residue in *A. californica* GluCl (Leu79) is not involved in its binding to glutamate or other amino acid agonists including GABA, suggesting that a role for the β 1 strand in agonist binding may be restricted to the *C. elegans* GluCl (Blarre *et al.*, 2014). Interestingly, replacement of *A. californica* GluCl Leu79 by Arg resulted in abolition of glutamate evoked currents, highlighting the importance of amino acids in the β 1 strand to receptor function. A comparison of GluCl structures in presumed open and closed conformations reveals that the β 1 and β 2 strands, as well as the β 1- β 2 loop, move towards the TM2-3 loop during gating (Althoff et al., 2014). This movement appears to precede the structural rearrangement of the TM domains that allow channel opening (Calimet et al., 2013). A similar conformational change has also been described in the recent structures of a zebrafish glycine receptor derived by cryo-electron microscopy (Du *et al.*, 2015).

Residues in the $\beta 1$ strand of the recently solved GABA_A $\beta 3$ homopentamer structure are not implicated in binding its agonist, benzamidine (Miller & Aricescu, 2014). However, residues in loop D of the anti-parallel $\beta 2$ strand are, and this region of the $\alpha 1$ subunit lies within the GABA binding pocket in $\alpha 1\beta 2\gamma 2$ GABA_A receptors (Boileau *et al.*, 1999; Holden & Czajkowski, 2002). Furthermore, the loop D residue Phe64 participates in gating (Szczot *et al.*, 2014). The use of the substituted Cys accessibility method (SCAM) revealed a series of $\beta 2$ strand residues within loop D that are accessible within the binding pocket. Consistent with its role in agonist binding, residue 64 became less accessible to methanethiosulfonate (MTS) modification when receptors were activated by GABA. It is likely that residues within the $\alpha 1$ subunit $\beta 1$ strand are also solvent accessible through the ligand binding pocket since they lie adjacent to the $\beta 2$ strand. As a result, Cys substituted $\beta 1$ strand residues may also be amenable to SCAM.

In this study, we first examined solvent access to the GABA_A $\beta 3$ homopentamer using molecular dynamics (MD) simulation. We subsequently used the simulations as a guide to investigate the functional consequences of Cys substitutions at five of the equivalent $\beta 1$ strand residues in the $\alpha 1$ subunit of $\alpha 1\beta 2\gamma 2$ GABA_A receptors. The introduction of Cys at these positions enabled us to examine their accessibility in the absence and presence of receptor activation.

Methods

Cell culture and transfection - Human embryonic kidney 293 (HEK-293) cells were maintained in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 100 $\mu\text{g ml}^{-1}$ penicillin and 100 units ml^{-1} streptomycin at 37°C and 5% CO_2 . Cells were seeded at low density in 35 mm dishes for electrophysiology. Transfections were performed by calcium phosphate precipitation, using 1 μg total cDNA per dish, as described previously (Baptista-Hon et al., 2013). cDNAs encoding wild type (WT) and mutant mouse GABA_A subunits were in the pRK5 mammalian expression vector. For heteromeric expression of GABA_A $\alpha 1\beta 2\gamma 2$ subunits, a 1:1:1 transfection ratio was used. cDNA encoding enhanced green fluorescence protein (in pEGFP vector, 0.1 μg) was included to identify successfully transfected cells using fluorescence microscopy. Cells were washed with media 16 h after transfection and used after 48 to 72 h. All tissue culture reagents were obtained from Invitrogen (Paisley, UK).

Mutagenesis of GABA_A $\alpha 1$ subunits – Single point mutations were performed by overlap extension polymerase chain reaction (PCR) (Heckman & Pease, 2007). PCR products were digested using SmaI restriction endonuclease and ligated into pRK5 vector. All mutagenesis reactions and ligations were verified using agarose gel electrophoresis and constructs were sequenced prior to functional characterisation (Genetics Core Services, University of Dundee). All PCR and molecular cloning reagents were obtained from Fermentas (Thermo-Fisher, Loughborough, UK).

Electrophysiology - The whole-cell configuration of the patch-clamp technique was used to record GABA-evoked currents from HEK-293 cells expressing WT $\alpha 1\beta 2\gamma 2$ GABA_A receptors or receptors containing Cys-substituted $\alpha 1$ subunits. Recording electrodes were fabricated from borosilicate glass capillaries, which when filled with intracellular solution had resistances of 1.3 – 2.3 M Ω for whole-cell recordings. The electrode solution contained (in mM): 140 CsCl, 2 MgCl₂, 1.1 EGTA, 3 Mg-ATP, 10 HEPES (pH 7.4 with CsOH). The extracellular solution contained (in mM): 140 NaCl, 4.7 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 10 HEPES, 10 glucose (pH 7.4 with NaOH). Cells were voltage clamped at an electrode potential of -60 mV. Currents were evoked by rapid application of GABA using the three-pipe Perfusion Fast Step system (Warner Instruments, CA, USA), as described previously (Baptista-Hon et al., 2013).

All electrophysiological data were recorded using an Axopatch 200B amplifier. Data were low pass filtered at 2 kHz, digitised at 20 kHz using a Digidata 1320A interface and acquired using pCLAMP8 software (all from Molecular Devices, CA, USA).

Substituted cysteine accessibility method (SCAM) – The cysteine sulfhydryl-specific modifying effect of a methanethiosulfonate (MTS) reagent was determined using 2-aminoethyl methanethiosulfonate (MTSEA) or phenylmethanethiosulfonate (PhMTS; Toronto Research Chemicals, Toronto, Ontario, Canada). Unless otherwise indicated, HEK-293 cells transfected with WT or Cys-substituted subunits were exposed repeatedly to an EC₅₀ concentration of GABA to evoke stable baseline currents ($I_{control}$). Freshly diluted MTSEA (up to 10 mM) was then applied episodically to cells prior to their GABA EC₅₀ exposure. Altered current amplitudes following MTSEA exposure reveals accessibility to sulfhydryl modification ($I_{modified}$). The extent of modification was measured as a percentage change:

$$\% \text{ change} = \frac{I_{modified} - I_{control}}{I_{control}} \times 100$$

Cells were subsequently exposed to the reducing agent dithiothreitol (DTT; 10 mM) to reverse modification by MTSEA.

Rates of MTSEA modification were measured using empirically determined concentrations of MTSEA, which produced cumulative changes in the amplitude of GABA-evoked currents that reached steady state within the duration of the recording. MTSEA was applied episodically for intervals between 0.1 s and 2 s, prior to a challenge with EC₅₀ GABA. In all cases, the steady-state current amplitude reached that of $I_{modified}$ when a saturating concentration of MTSEA was used, indicating that the modification reaction had reached completion. To determine whether the rate of MTSEA modification of the substituted cysteine can be altered in the presence of agonists or allosteric agonists, a maximal concentration of GABA (EC₁₀₀), or an activating concentration of propofol (10 μM) was simultaneously applied with MTSEA.

MD simulations - MD simulation was carried out with the Gromacs simulation software version 4.67 (Pronk *et al.*, 2013). The ambersb99_ildn force field (Hornak *et al.*, 2006; Lindorff-Larsen *et al.*, 2010) for amino acids and the Berger force field for POPC lipids (Berger *et al.*, 1997) were applied. Temperature and pressure were kept constant at 298 K and 1 bar, using the v-rescale thermostat (Bussi *et al.*, 2007) and the Parrinello-Rahman barostat (Parrinello & Rahman, 1981) with coupling constants of 0.5 ps and 2 ps, respectively. Electrostatic interactions were computed using the Particle Mesh Ewald (PME) method with a real space cut-off of 12 Å. Van der Waals (VdW) interactions were calculated using a cut-off of 12 Å. The LINCS algorithm (Hess *et al.*, 1997) was applied to constrain all bonds. Virtual sites (Feenstra *et al.*, 1999) were employed for hydrogen atoms permitting us to use a simulation timestep of 5 fs. We simulated two receptor/membrane systems. The total sampling time was 1.64 μs.

pK_a values of all titrable groups of the GABA_A β3 homopentamer (PDB-ID: 4COF) (Miller & Aricescu, 2014) were calculated using the H++ webserver (Anandakrishnan *et al.*, 2012) and checked manually. The transmembrane domain of the protein was first aligned to a POPC bilayer, using the program LAMBADA (Schmidt & Kandt, 2012) and slightly moved manually to match protein-lipid interactions (Contreras *et al.*, 2011). The protein was inserted into the membrane, employing the program InflateGRO2 (Schmidt & Kandt, 2012). A NaCl concentration of ~180 mM was added and additional counter ions were introduced to neutralize the simulation system.

Data analysis - The peak amplitudes of agonist-evoked currents were measured using Clampfit10 software (Molecular Devices, CA, USA). Individual relationships of current amplitude to GABA concentration were fitted with a logistics equation:

$$f([GABA]) = \frac{100}{1 + 10^{(\log EC_{50} - [GABA]) \times n^H}}$$

From which GABA EC₅₀ and Hill slope (n^H) values were determined.

The rate of MTSEA modification of Cys substituted receptors was measured by fitting single or double exponential functions to peak current amplitude data following cumulative MTSEA applications. The double exponential function is defined by:

$$f(t) = Plateau + A_f e^{-t/\tau_f} + A_s e^{-t/\tau_s}$$

Where τ_f and τ_s represent the fast and slow time constants, respectively. A_f and A_s represent the proportion of the fast and slow components, respectively, such that A_f and A_s sum to 1 - plateau. Rate of MTSEA modification are provided as weighted τ (τ_w) values, calculated using:

$$\tau_w = A_f \times \tau_f + A_s \times \tau_s$$

Pseudo first-order rate constants were derived from a modified single or double exponential fit to peak current amplitude data following MTSEA applications. The double exponential function is defined by:

$$f(t) = Plateau + A_f \times e^{(K_f \times t)} + A_s \times e^{(K_s \times t)}$$

Where K_f and K_s represent the fast and slow first-order rates, respectively. A_f and A_s represent the proportion of the fast and slow components. Since all amplitude data are normalised to I_{GABA} at t = 0, A_f and A_s sum to 1 - plateau. Second-order rate constants were obtained by dividing K by the concentration of MTSEA used (Holden & Czajkowski, 2002).

The solvent accessible surface area (SASA) was calculated as described previously (Eisenhaber *et al.*, 1995) using data from the MD simulations. The solvent accessible area was graphically represented using `trj_cavity` (Paramo *et al.*, 2014). All figures containing molecular information were produced with VMD (Humphrey *et al.*, 1996).

Statistics - Data are presented as mean \pm S.E.M. or S.D. as indicated. Differences in means of three or more groups were compared using one-way analysis of variance (ANOVA), with a *post hoc* Tukey or Dunnett's test, as appropriate. Pairwise comparisons were performed using the student t-test. In all cases $P < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, California, U.S.A.).

Results

Residues in the β 1 strand of the β 3 homopentamer are solvent accessible

Changes in the accessibility of residues during GABA_A receptor activation have been determined using SCAM (Boileau et al., 1999). Two criteria are required for successful SCAM. First, the substituted Cys must be solvent accessible and second it must participate in a receptor function that is affected by modification. Both of these criteria apply to the GABA_A receptor α 1 subunit β 2 strand loop D residue at position 64. This residue is solvent accessible and its modification by MTS reagents led to a reduction in GABA-evoked current amplitudes in receptors harbouring the α 1(F64C) substitution (Boileau et al., 1999). Phe64 participates in GABA binding and efficacy (Boileau et al., 1999; Szczot et al., 2014). While residues in the β 1 strand do not participate in agonist binding to the GABA_A β 3 homopentamer structure, we examined whether they are likely to be accessible through the binding pocket (Miller and Aricescu, 2014). We performed MD simulations of the whole protein but focused our analysis on residues located within the β 1 strand of the β 3 homopentamer (Fig. 1A).

Our simulations reveal that a number of residues in the β 1 strand of the GABA_A β 3 homopentamer are solvent accessible. A representative snapshot of the access pathways of water to the agonist binding site observed in the simulations is shown in Figure 1B. Consistent with previous reports, the critical β 2 strand loop D residue, equivalent to 64 in the α 1 subunit, which is Tyr62 in the β 3 homopentamer, is also accessible. We quantified the solvent accessible surface area (SASA) for these residues (Fig. 1C). The SASA values for residues from 41 to 45 are characteristic of a β -strand structure with an alternating pattern of accessibility. While residues Asn41, Asp43 and Ala45 display significant solvent accessibility, Ile42 and Ile44 are inaccessible. The number of water molecules observed to be in contact with the residues throughout our simulations agrees well with their SASA (data not shown). Other residues, e.g. Cys37, Ser46, Asp48, Met49 and Ser51 also have significant SASA values and may therefore be accessible. However, inspection of the model reveals that the highly accessible Cys37 and Met49 are located outside the binding pocket at the surface of the receptor and within the outer vestibule, respectively. Furthermore, in the β 3 homopentamer model, the β 1 strand has a sharp kink after residue 45, dipping towards the vestibule.

β 1 strand residues influence the apparent potency of GABA

Our MD simulations (Fig. 1) suggest that several residues within the β 1 strand of GABA_A receptors are part of a solvent accessible pocket contiguous with the ligand binding domain. We investigated the functional consequences of individual Cys substitutions from Asp43 to Thr47 in the α 1 subunit. These residues are homologous to those between Asn41 and Ala45 in the GABA_A β 3 homopentamer

according to our sequence alignment (Fig. 1A). For comparison, we also included Cys-substituted Phe64 in loop D of the $\beta 2$ strand in our analysis. WT or Cys substituted $\alpha 1$ subunits were transiently expressed in HEK-293 cells with $\beta 2$ and $\gamma 2$ subunits. Representative examples of GABA-evoked currents mediated by Cys substituted GABA_A receptors are shown in Figure 2A. GABA concentration-response relationships are plotted in Figure 2B. Logistic functions fitted to the data reveal rightward shifts in the GABA concentration-response relationship for some Cys-substituted receptors. The average best-fit parameters derived from logistic functions fitted to data recorded from several different cells are summarised in Table 1. Consistent with previous reports, $\alpha 1(F64C)\beta 2\gamma 2$ receptors displayed a large increase in EC_{50} ($P < 0.0001$; t-test vs WT $\alpha 1\beta 2\gamma 2$ receptors), confirming an important role for this $\beta 2$ strand residue in GABA_A receptor function. For $\beta 1$ strand Cys-substituted $\alpha 1\beta 2\gamma 2$ receptors, one-way ANOVA with a Dunnet's comparison revealed a significant difference in GABA EC_{50} between $\alpha 1(D43C)\beta 2\gamma 2$ and WT $\alpha 1\beta 2\gamma 2$ receptors ($P < 0.0001$; Table 1). $\alpha 1(I44C)\beta 2\gamma 2$ and $\alpha 1(T47C)\beta 2\gamma 2$ receptors showed tendencies towards increased EC_{50} values, but these were not significantly different to WT. $\alpha 1(V46C)\beta 2\gamma 2$ receptors had EC_{50} values indistinguishable from WT $\alpha 1\beta 2\gamma 2$ receptors. The Hill slope values for these Cys substituted GABA_A receptors were similar to that of WT $\alpha 1\beta 2\gamma 2$ receptors (Table 1). Like $\alpha 1(F64C)\beta 2\gamma 2$, both $\alpha 1(D43C)\beta 2\gamma 2$ and $\alpha 1(T47C)\beta 2\gamma 2$ receptors had reduced GABA-evoked peak current densities compared to WT which were statistically significant ($P < 0.0001$ and $P < 0.05$ respectively; one-way ANOVA with *post hoc* Dunnet's comparison with $\alpha 1\beta 2\gamma 2$ receptors; Table 1). The Cys substitution in $\alpha 1(F45C)\beta 2\gamma 2$ receptors produced more complicated changes in function described in the following section.

The $\alpha 1$ F45C substitution causes a biphasic GABA concentration-response relationship and spontaneous gating

The GABA concentration-response relationship for $\alpha 1(F45C)\beta 2\gamma 2$ receptors is shown in Figure 3A, with representative examples for GABA-evoked currents shown in the inset. A single component logistic function provided an inadequate representation of the concentration-response relationship for GABA-evoked currents mediated by $\alpha 1(F45C)\beta 2\gamma 2$ receptors. By contrast, a two-component logistic function provided a good fit across the entire GABA concentration range (solid black line; Fig. 3A). An F-test applied to statistically discriminate between the two approaches revealed that the two-component logistic function provided a significantly improved fit to the data ($P = 0.025$). The solid grey lines in Figure 3A illustrate the two components of the GABA concentration-response relationship. The logistic fit for WT $\alpha 1\beta 2\gamma 2$ receptors is also reproduced here (dashed grey line) for comparison. Table 1 contains the values derived from the two-component logistic function

consistent with high and low apparent potency components for GABA-evoked activation of $\alpha 1(F45C)\beta 2\gamma 2$ receptors.

GABA_A receptors with high levels of spontaneous gating generally have a higher sensitivity to GABA (Mortensen *et al.*, 2003; Hadley & Amin, 2007). The high apparent potency component of GABA activation of $\alpha 1(F45C)\beta 2\gamma 2$ receptors may therefore be associated with spontaneous activity. We investigated the possibility of spontaneous gating by recording the inhibition of basal currents by picrotoxin (PTX) in WT $\alpha 1\beta 2\gamma 2$ and $\alpha 1(F45C)\beta 2\gamma 2$ receptors. Figure 3B shows representative examples of maximally effective GABA-evoked currents (grey trace) and inhibition of basal currents by PTX (black trace). WT $\alpha 1\beta 2\gamma 2$ receptors display very little PTX inhibited basal current (Fig. 3B inset). By comparison, the magnitude of PTX inhibited basal current in $\alpha 1(F45C)\beta 2\gamma 2$ receptors was large (Fig. 3B inset). We quantified spontaneous current as the magnitude of the PTX component (I_{PTX}) as a percentage of the total amount of current ($I_{GABA} + I_{PTX}$). The maximum GABA-evoked current densities in WT $\alpha 1\beta 2\gamma 2$ and $\alpha 1(F45C)\beta 2\gamma 2$ receptors did not differ significantly (Table 1). Therefore it is unlikely that the maximum efficacy of GABA is altered by the F45C substitution. The mean I_{PTX}/I_{total} values are shown in Figure 3C. $\alpha 1(F45C)\beta 2\gamma 2$ receptors had an increased proportion of spontaneous current from $0.05 \pm 0.02\%$ ($n = 5$) in WT $\alpha 1\beta 2\gamma 2$ receptors to $0.52 \pm 0.11\%$ ($n = 10$). The difference was statistically significant ($P = 0.014$; t-test). These data suggest that F45C substitution increased spontaneous gating and therefore Phe45 in the $\beta 1$ strand plays critical and complex roles in GABA_A receptor function.

Several residues in the $\beta 1$ strand of the GABA_A $\alpha 1$ subunit are accessible to MTSEA modification

$\beta 1$ strand residues in the GluCl α subunit (Hibbs & Gouaux, 2011) and GABA_A $\beta 3$ homopentamer (Miller & Aricescu, 2014) models have side-chains facing into the orthosteric binding pocket. Our MD simulation data demonstrate that residues in the GABA_A $\beta 3$ homopentamer, equivalent to amino acids 43 – 47 in the $\alpha 1$ subunit, are solvent accessible (Fig. 1C). Furthermore, receptors containing $\alpha 1$ subunit D43C, F45C, T47C substituents all exhibit functions distinguishable from WT receptors (Figs 2 and 3 and Table 1). Therefore, $\alpha 1$ subunit $\beta 1$ strand residues may be accessible to MTS reagents and their modification may alter function.

We used SCAM to investigate whether the substituted cysteine residues in the $\beta 1$ strand of the GABA_A receptor are accessible to the sulfhydryl reagent, MTSEA. The use of a similar approach previously in *Xenopus* oocytes demonstrated accessibility of substituted cysteines in $\alpha 1(F64C)$ containing GABA_A receptors (Boileau *et al.*, 1999; Holden & Czajkowski, 2002). We adapted the

technique for use in whole-cell patch-clamp recordings from HEK-293 cells using rapid application of GABA and MTSEA (see Methods), and used $\alpha 1(F64C)\beta 2\gamma 2$ receptors for comparison.

We first tested whether MTSEA (10 mM) modulates GABA_A receptors containing WT $\alpha 1$ subunits (Fig. 4A). Control currents were evoked by an EC₅₀ concentration of GABA (10 μ M) in HEK-293 cells expressing $\alpha 1\beta 2\gamma 2$ GABA_A receptors. Modification by MTSEA (10 mM) was tested by pre-application for 2 s prior to GABA. Five applications were tested, such that cells were exposed to MTSEA cumulatively for 10 s. The amplitudes of GABA-evoked currents appeared modestly enhanced following MTSEA treatment in some cells, but the mean did not significantly differ from that of controls (n = 6). We next confirmed that MTSEA (2 mM) modifies the function of $\alpha 1(F64C)\beta 2\gamma 2$ receptors. MTSEA substantially reduced the current amplitude evoked by an EC₅₀ concentration of GABA (10 mM; Fig. 4A). The application of the reducing agent DTT (10 mM) reversed the modification by MTSEA. This result is consistent with a previous report of the effects of SCAM on GABA_A receptors containing the $\alpha 1(F64C)$ subunit (Boileau et al., 1999).

Using the same approach, we applied MTSEA (10 mM) to GABA_A receptors containing Cys-substituted $\beta 1$ strand residues. Exemplar currents evoked by an EC₅₀ concentration of GABA before and after MTSEA application are shown in Figure 4B. MTSEA application caused a reduction in GABA-evoked current amplitude in $\alpha 1(D43C)\beta 2\gamma 2$ and $\alpha 1(T47C)\beta 2\gamma 2$ receptors. MTSEA did not affect the amplitude of $\alpha 1(I44C)\beta 2\gamma 2$, $\alpha 1(F45C)\beta 2\gamma 2$ or $\alpha 1(V46C)\beta 2\gamma 2$ receptors. The equivalent residues to Cys44 and Cys46 in the GABA_A $\beta 3$ homopentamer showed low SASA values and the failure of MTSEA to affect function is consistent with their lack of solvent accessibility (Fig. 1C).

It is interesting that $\alpha 1(F45C)\beta 2\gamma 2$ receptors were unaffected by MTSEA despite a high SASA (Fig. 1C) and the importance of the identity of this residue in receptor function (Fig. 3). It is possible that modification by MTSEA, which would add a positive charge to the Cys residue, was functionally silent in these receptors. We therefore used a different sulfhydryl modifying reagent, phenylmethanesulfonate (PhMTS) which adds an aromatic group to an accessible Cys, a modification that would mimic the native Phe. However, PhMTS (200 μ M) applied for over 2 min also had no effect on the function of $\alpha 1(F45C)\beta 2\gamma 2$ receptors (data not shown). In addition, we repeated the MTSEA experiment on $\alpha 1(F45C)\beta 2\gamma 2$ receptors using 1 mM GABA as the test concentration. The effect of 1 mM GABA was maximal at WT $\alpha 1\beta 2\gamma 2$ receptors (Fig. 2C), whereas this concentration was only 80% effective at $\alpha 1(F45C)\beta 2\gamma 2$ receptors (Fig. 3A). However, MTSEA had no effect on the amplitude of GABA (1 mM)-evoked currents (data not shown). In addition to an altered GABA concentration-response relationship, $\alpha 1(F45C)\beta 2\gamma 2$ receptors displayed higher

spontaneous activity compared to WT $\alpha 1\beta 2\gamma 2$ receptors. We therefore examined whether MTSEA could influence the extent of spontaneous activity mediated by $\alpha 1(F45C)\beta 2\gamma 2$ receptors. Cells were treated with MTSEA (2 mM) for 2 min and spontaneous activity was measured as the percentage of I_{PTX} to I_{total} . Figure 4C compares I_{PTX}/I_{total} in control conditions and in the presence of MTSEA (2 mM). There was no significant difference in the level of spontaneous activity in the presence of MTSEA. Taken together, these data suggest that, while Cys43 and Cys47 are accessible to MTSEA, Cys45 is inaccessible.

We quantified the extent of MTSEA modification determining the percentage changes in EC_{50} GABA-evoked current amplitude for each Cys substituted receptor before and after MTSEA application. These values are plotted in Figure 4D. Application of MTSEA caused a significant reduction in the amplitudes of GABA-evoked currents mediated by $\alpha 1(D43C)\beta 2\gamma 2$, $\alpha 1(T47C)\beta 2\gamma 2$ and $\alpha 1(F64C)\beta 2\gamma 2$ receptors relative to those mediated by WT receptors ($P < 0.0001$; one-way ANOVA; *post hoc* Dunnet's comparison vs $\alpha 1\beta 2\gamma 2$).

Receptor activation influences MTSEA modification of D43C and T47C containing receptors

Receptor activation by an agonist might influence the accessibility of a substituted Cys. This could occur as a result of the bound agonist directly protecting the residue from modification. Alternatively activation may cause a conformational rearrangement of the receptor in which the Cys becomes less accessible. The $\alpha 1(F64C)$ was modified more slowly by an MTS reagent applied with GABA to *Xenopus* oocytes expressing $\alpha 1(F64C)\beta 2\gamma 2$ receptors (Boileau et al., 1999). We investigated whether GABA also affects the modification of $\alpha 1(D43C)\beta 2\gamma 2$ and $\alpha 1(T47C)\beta 2\gamma 2$ receptors by applying MTSEA in its presence and absence. Once again we modified the approach for use in voltage-clamped HEK-293 cells (see Methods).

First, using this approach, we compared the rate of MTSEA modification of $\alpha 1(D43C)\beta 2\gamma 2$, $\alpha 1(T47C)\beta 2\gamma 2$ and $\alpha 1(F64C)\beta 2\gamma 2$ receptors. Figure 5A shows representative examples of the effect of rapid and short applications of MTSEA on GABA-evoked currents mediated by $\alpha 1(D43C)\beta 2\gamma 2$, $\alpha 1(T47C)\beta 2\gamma 2$ and $\alpha 1(F64C)\beta 2\gamma 2$ receptors. The concentration of MTSEA used for each mutant was chosen empirically on the basis of a measurable reduction in the amplitude of the EC_{50} GABA-evoked current during the time course of the experiment. For $\alpha 1(D43C)\beta 2\gamma 2$ and $\alpha 1(F64C)\beta 2\gamma 2$ receptors, MTSEA (100 μ M and 10 μ M, respectively) was applied episodically for 100 ms, prior to application of a test concentration of GABA (EC_{50}). For both receptors, the modification was complete within 500 ms. The rate of MTSEA (100 μ M) modification of $\alpha 1(T47C)\beta 2\gamma 2$ receptors was slower and therefore MTSEA was applied episodically for 500 ms prior to the application of GABA (EC_{50}). We analysed the

change in EC₅₀ GABA-evoked current amplitude and expressed these as a percentage of the control current. Figure 5B shows the rate of MTSEA evoked reduction of GABA-evoked currents mediated by $\alpha 1(\text{D43C})\beta 2\gamma 2$, $\alpha 1(\text{T47C})\beta 2\gamma 2$ and $\alpha 1(\text{F64C})\beta 2\gamma 2$ receptors. In all cases, the time course of MTSEA modification was well fitted with a single exponential. The mean (\pm S.E.M.) time constants of modification are plotted in Figure 5C. One-way ANOVA with a *post hoc* Tukey's comparison of the time constants revealed that both $\alpha 1(\text{D43C})\beta 2\gamma 2$ and $\alpha 1(\text{F64C})\beta 2\gamma 2$ receptors exhibited modification time constants which were significantly smaller than that of $\alpha 1(\text{T47C})\beta 2\gamma 2$ receptors ($P < 0.0001$). We also determined the second-order rate constants for MTSEA modification (see methods). Second-order rate constants are summarised in Table 2. The second-order rate constants for $\alpha 1(\text{F64C})\beta 2\gamma 2$ receptors were significantly faster than that of $\alpha 1(\text{D43C})\beta 2\gamma 2$ and $\alpha 1(\text{T47C})\beta 2\gamma 2$ receptors ($P < 0.0001$; one-way ANOVA; *post hoc* Tukey's comparison; Table 2). Our second-order rate constant for MTSEA modification, derived from HEK cells expressing $\alpha 1(\text{F64C})\beta 2\gamma 2$ receptors, is similar to that reported by Holden and Czajkowski (2002) in oocytes. The plateau of modification for $\alpha 1(\text{D43C})\beta 2\gamma 2$, $\alpha 1(\text{T47C})\beta 2\gamma 2$ and $\alpha 1(\text{F64C})\beta 2\gamma 2$ receptors, which indicates the maximal extent in functional change for the concentration of MTSEA tested, did not differ from those shown in Figure 4D, which used a high concentration of MTSEA (2 mM). This demonstrates that modification was complete. Our data suggest that Cys64 and Cys43 are more accessible to modification than Cys47.

We next repeated the experiment in the presence of either a maximal concentration of GABA, or an activating concentration of propofol (10 μM), applied simultaneously with MTSEA. In all cases ($\alpha 1(\text{F64C})\beta 2\gamma 2$, $\alpha 1(\text{D43C})\beta 2\gamma 2$ and $\alpha 1(\text{T47C})\beta 2\gamma 2$ receptors), the co-application of either GABA or propofol with MTSEA induced inward currents. Figure 6A shows the rate of MTSEA (10 μM) modification of $\alpha 1(\text{F64C})\beta 2\gamma 2$ receptors in the presence of either GABA (300 mM) or propofol (10 μM). The rate of modification was again measured by episodic (100 ms) application of MTSEA (10 μM) with GABA (300 mM) or propofol (10 μM) for the first 500 ms. Propofol did not affect the rate of MTSEA modification (Fig. 6A inset). In the presence of GABA (300 mM) however, the extent of modification following 500 ms of MTSEA exposure was less than in its absence (Fig. 6A inset). We therefore increased the duration of each episodic application to 2 s. The rate of modification in the presence of GABA was best fitted with a double exponential (Fig. 6A). The weighted τ was calculated for modification when MTSEA was applied simultaneously with GABA and compared with the τ for modification in the absence of GABA. The mean (\pm S.E.M.) time constants are plotted in Figure 6B. The second-order rate constants derived from the exponential fits are summarised in Table 2. GABA (300 mM), but not propofol (10 μM), significantly slowed the rate of MTSEA (10 μM) modification at $\alpha 1(\text{F64C})\beta 2\gamma 2$ receptors ($P = 0.0004$; one-way ANOVA; *post hoc* Tukey's comparison). Our data are

consistent with previous observations indicating that GABA, but not the allosteric agonist pentobarbital, influences modification of $\alpha 1(\text{F64C})$ containing GABA_A receptors (Boileau *et al.*, 1999). Using the same approach, we determined the effect of GABA and propofol on MTSEA (100 μM) modification of $\alpha 1(\text{D43C})\beta 2\gamma 2$ and $\alpha 1(\text{T47C})\beta 2\gamma 2$ receptors. The concentrations of GABA used were 300 mM and 30 mM, respectively and 10 μM propofol was used for both receptors. The time course for MTSEA modification in control, in the presence of GABA and in the presence of propofol for $\alpha 1(\text{D43C})\beta 2\gamma 2$ receptors is shown in Figure 6D. MTSEA in the presence or absence of agonists was applied cumulatively for 100 ms. The rates of modification in all three conditions were well fitted with single exponential functions (Fig. 6D). The mean (\pm S.E.M.) time constants and extent of functional change are plotted in Figure 6E and F, respectively. Second-order rate constants are summarised in Table 2. In the presence of GABA (300 mM), there was a significant increase in the τ of MTSEA modification ($P < 0.05$; one-way ANOVA; *post hoc* Tukey's comparison vs control). There was a tendency for the τ of modification to increase in the presence of propofol, but this was not statistically significant. However, there was also no significant difference in the τ of modification in the presence of GABA and in the presence of propofol. Furthermore, comparison of the extent of functional change revealed a statistically significant reduction between that in the presence of GABA and control ($P < 0.05$; one-way ANOVA; *post hoc* Tukey's comparison vs control), and that in the presence of propofol and control ($P < 0.05$; one-way ANOVA; *post hoc* Tukey's comparison vs control; Fig. 6F). These data therefore suggest that both GABA and propofol influence MTSEA modification of Cys43. This is in contrast to Cys64 in loop D where the allosteric agonist propofol did not influence MTSEA modification (Fig. 6A-C).

Figure 6G shows the time course of MTSEA (100 μM) modification of $\alpha 1(\text{T47C})\beta 2\gamma 2$ receptors in control, in the presence of GABA (30 mM) and in the presence of propofol (10 μM). MTSEA in the presence or absence of agonists was applied cumulatively for 500 ms. The time course for modification in each case was well fitted with a single exponential function. The mean (\pm S.E.M) τ of modification and the extent of functional change are plotted in Figure 6H and I respectively. Second-order rate constants are summarised in Table 2. GABA (30 mM) and propofol (10 μM) did not significantly alter the τ of MTSEA modification at $\alpha 1(\text{T47C})\beta 2\gamma 2$ receptors. However, both GABA and propofol significantly reduced the extent of MTSEA mediated inhibition ($P < 0.05$; one-way ANOVA; *post hoc* Tukey's comparison vs control; Fig. 6I).

Discussion

In this study, we investigated the role of the $\alpha 1$ subunit $\beta 1$ strand in GABA_A receptor function. Guided by MD simulation data on the SASA of equivalent residues of the GABA_A $\beta 3$ homopentamer, we mutated five continuous residues on the $\beta 1$ strand of the GABA_A $\alpha 1$ subunit to Cys. Whole-cell voltage clamp electrophysiology revealed that D43C, F45C and T47C substitutions caused changes in the GABA concentration-response relationship, demonstrating for the first time that the $\beta 1$ strand residues play a role in mammalian pLGIC function.

The $\beta 1$ strand runs adjacent and parallel to the critical $\beta 2$ strand, which contains loop D. A crucial Phe (Phe64) in the $\beta 2$ strand of the GABA_A receptor $\alpha 1$ subunit plays a critical role in both GABA binding and the conformational transition which leads to orthosteric gating of the channel (Boileau *et al.*, 1999; Szczot *et al.*, 2014). These effects also manifest as a large rightward shift in the apparent potency for GABA. The agonist concentration-response relationship (and therefore apparent potencies) are composites of binding and gating events, and therefore both can influence the concentration-response relationship (Colquhoun, 1998). Indeed, $\alpha 1$ subunit Phe64 in the $\beta 2$ strand, which causes large rightward shifts in the apparent potency of GABA, when Cys substituted, is an example of a residue with a dual action in binding and gating efficacy (Boileau *et al.*, 1999; Szczot *et al.*, 2014). Substituted Cys64 in the GABA_A receptor $\alpha 1$ subunit was accessible to sulfhydryl modification when expressed in *Xenopus* oocytes (Boileau *et al.*, 1999; Holden & Czajkowski, 2002). Using fast applications of MTSEA, we showed that the same substitution in $\alpha 1\beta 2\gamma 2$ receptors expressed in HEK-293 cells was modified with a similar second-order rate constant. Furthermore, as previously reported, the presence of GABA slows the rate of Cys64 modification while the allosteric agonist propofol had no effect. This is in contrast to $\alpha 1(D43C)\beta 2\gamma 2$ and $\alpha 1(T47C)\beta 2\gamma 2$ receptors, where both GABA and propofol affect MTSEA mediated modification of the substituted Cys, suggesting that $\beta 1$ strand residues play a different role to that of Phe64 in the $\beta 2$ strand in terms of GABA_A receptor activation.

Changes in Cys accessibility during receptor activation can be due to either direct or indirect effects of agonists. Direct agonist effects likely arise from hindrance of the substituted Cys by the bound agonist (protection), while indirect effects likely arise from conformational changes induced by the agonist which alter the position of the substituted Cys, making it less accessible. Our data show that an activating concentration of propofol impairs the accessibility of both Cys43 and Cys47 in a similar manner to a maximally effective concentration of GABA. This is consistent with a conformational change associated with gating resulting in reduced accessibility of positions 43 and 47. This also suggests that the structural rearrangements in the $\beta 1$ strand of the GABA_A receptor $\alpha 1$ subunit is

similar, irrespective of agonist binding in the vicinity, or far away from the orthosteric binding site. A conformational change in this region makes sense given the anti-parallel location of the $\beta 1$ relative to the $\beta 2$ strand, together constituting a hairpin structure that participates in the transduction of binding to GABA_A channel opening. Indeed, Kash *et al* (2003) implicated an interaction between the loop connecting the $\beta 1$ and $\beta 2$ strands of the $\alpha 1$ subunit, and the TM2-3 loop during GABA_A receptor activation. Furthermore, we have previously demonstrated that substitution of the conserved $\alpha 1$ subunit TM2-3 Lys278 with methionine reduces the efficacy of activation by GABA and propofol (Hales *et al.*, 2006). The same mutation also reduced the level of spontaneous gating of $\alpha 1(K278M)\beta 2\gamma 2$ GABA_A receptors consistent with a global reduction in gating (Othman *et al.*, 2012). Comparison of the open (ivermectin and glutamate bound) and closed (*apo*) structures of GluCl also reveals movement in this region associated with channel opening (Althoff *et al.*, 2014). During activation there is a downward displacement of the $\beta 1$ - $\beta 2$ loop, increasing its proximity to the TM2-3 loop. The agonist bound and apo-GluCl models also reveal large movements of residues within the $\beta 1$ and $\beta 2$ strands, particularly Arg37 in the $\beta 1$ strand, homologous to Thr47 in this study. Comparison of the open (glycine bound) and closed (strychnine bound) structures of the zebrafish glycine receptor reveal similar movements of the $\beta 1$ - $\beta 2$ loop (Du *et al.*, 2015). It is therefore becoming evident that residues along the $\beta 1$ and $\beta 2$ strands are well positioned to participate in the transduction of agonist binding to gating.

While $\alpha 1(D43C)\beta 2\gamma 2$ and $\alpha 1(T47C)\beta 2\gamma 2$ receptors were sensitive to MTSEA modification, $\alpha 1(I42C)\beta 2\gamma 2$, $\alpha 1(F45C)\beta 2\gamma 2$ and $\alpha 1(V46C)\beta 2\gamma 2$ receptors were not. The low SASA values of the homologous residues to those in position 42 and 46 of the GABA_A $\beta 3$ homopentamer (Ile42 and Ile44 respectively; Fig. 1) suggest that these residues are not solvent exposed, but instead have their side chains orientated within a hydrophobic environment. However, the homologous position to Cys45 in the GABA_A $\beta 3$ homopentamer (Asp43) is solvent accessible; this contrasts with our SCAM data which demonstrate that Cys45 is not modifiable by MTSEA or PhMTS. We propose that the carboxylate group of Asp43 (equivalent to Phe45 in $\alpha 1$) causes an increased hydration of the agonist binding site in the case of the $\beta 3$ homopentamer, as it has previously been shown that negatively charged side chains can promote solvation even in highly hydrophobic regions (Krah *et al.*, 2010; Villinger *et al.*, 2010). The structure of the $\alpha 1$ subunit within the heteromeric $\alpha 1\beta 2\gamma 2$ receptor may also differ significantly from the $\beta 3$ subunit altering the environment of $\alpha 1$ residue 45 relative to the equivalent $\beta 3$ residue 43. The most obvious structural difference between these two subunits in the vicinity of this residue is in loop F, which together with loop D sandwich the $\beta 1$ strand. Loop F has two fewer residues in the $\alpha 1$ subunit compared to the $\beta 3$ subunit.

Our data also demonstrate that $\alpha 1(F45C)\beta 2\gamma 2$ receptors have a biphasic GABA concentration-response relationship with increased spontaneous gating. The component of the biphasic GABA concentration-response relationship, with the higher apparent potency, may occur as a consequence of increased spontaneous gating observed for $\alpha 1(F45C)\beta 2\gamma 2$ relative to WT receptors. Our finding that $\alpha 1(F45C)\beta 2\gamma 2$ exhibits increased spontaneous gating suggests that residue Phe45 stabilises the receptor in the inactive conformation.

Mutations that increase spontaneous gating typically cause a left-shifted concentration-response relationship, where receptors have a higher sensitivity to agonists (Mortensen *et al.*, 2003; Hadley & Amin, 2007). The component of the $\alpha 1(F45C)\beta 2\gamma 2$ concentration-response relationship, with a lower apparent potency, has a shallow Hill coefficient (~ 0.5 ; Fig. 3A; Table 1), suggesting that cooperativity between adjacent GABA binding sites is impaired. It is worth considering how residue 45 in the first occupied orthosteric site may participate in communication with the subsequently occupied binding site. As mentioned previously, the $\beta 1$ strand lies between loop D on the $\beta 2$ strand and loop F. Loop F in GABA_A $\rho 1$ homopentamers and $\alpha 1\beta 2\gamma 2$ heteromers has been implicated in cooperativity of binding and gating, respectively. Site-directed fluorescence spectroscopy of GABA_A $\rho 1$ receptors, implicated structural rearrangements in loop F during orthosteric ligand binding, which were unrelated to gating (Khatri *et al.*, 2009). Interestingly, for GABA_A $\rho 1$ receptors, a number of loop F Cys substitutions appeared to reduce the Hill coefficient. Loop F residues in the $\gamma 2$ subunit are also implicated in transducing benzodiazepine binding events to altered gating (Hanson & Czajkowski, 2008) and it has been postulated that loop F residues play a role in cooperativity of agonist binding (Khatri & Weiss, 2010). Therefore, Phe45 in the $\beta 1$ strand of the GABA_A $\alpha 1$ subunit may transduce structural rearrangements within the binding pocket to the adjacent binding domain, via loop F. Therefore, the lack of Phe45 in the $\alpha 1(F45C)\beta 2\gamma 2$ receptor may lead to an uncoupling of binding between the two orthosteric binding sites. In addition, the existence of receptors in either the spontaneously open conformation or in the closed state may provide an explanation for high and low sensitivity components of the concentration response relationship.

Residues in the $\beta 1$ and $\beta 2$ strands of the *Erwinia chrysanthemi* ligand gated ion channel (ELIC) (Pan *et al.*, 2012) and neuronal $\alpha 7$ acetylcholine receptors (Quiram *et al.*, 2000) have been implicated in the recognition of antagonists. The *C. elegans* GluCl α subunit homopentamer is the only structure which implicates $\beta 1$ strand residues in agonist binding, and consequently this region was postulated to be a seventh ligand binding loop, termed loop G (Hibbs & Gouaux, 2011). Arg45 in the *C. elegans* GluCl α subunit appears to participate in glutamate binding. By contrast, $\beta 1$ strand residues of the GABA_A $\beta 3$ (Miller & Aricescu, 2014), and the 5-HT_{3A} receptors (Hassaine *et al.*, 2014), lie outside the

ligand binding site. Emerging structural data suggest that a direct role for β 1 strand residues in agonist binding may be restricted to the *C. elegans* GluCl (Blarre *et al.*, 2014).

Our findings suggest that naturally occurring mutations and polymorphisms that affect the β 1 strand may result in functional deficits that could be pathological. We are unaware of any such mutations that have been identified to date, but knowledge of the importance of this region of the receptor may prove to be important in future studies.

Refinement of the SCAM for use with rapid solution exchange should enable an investigation of altered accessibility caused by specific states of the receptor induced by agonist activation. We anticipate that this approach will help bridge the gap between static structural models and real time recordings of electrophysiology. This will help to discriminate between conformational changes caused by activation and desensitisation.

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Additional Information

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Author contributions – D.T.B-H, U.Z. and T.G.H. designed the work. D.T.B-H and A.K. acquired the data. D.T.B-H, A.K., U.Z. and T.G.H. analysed and interpreted the data. D.T.B-H, A.K., U.Z. and T.G.H. drafted the work and revised it critically for intellectual content. All authors approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions relating to the accuracy and integrity of the work are appropriately investigated and resolved. All authors qualify for authorship and only those who qualify for authorship are listed.

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Figure legends

Figure 1. Amino acids implicated in agonist binding to GABA_A and GluCl receptors in β 1 and β 2 strands, respectively. **A**, Amino acid sequence alignment of the mouse GABA_A α 1 and β 3 subunits, and *C. elegans* GluCl α subunit. The region shown in the sequence alignment contains the residues relevant to this study. Residues in the GABA_A β 3 subunit evaluated using MD simulation are in black. The residues in the β 1 strand of the α 1 subunit mutated to Cys are in bold and underlined. **B**, Graphical representation of the solvent accessible area of the GABA_A β 3 homopentamer from a representative snapshot. The β 1 strand is shown in blue in all five subunits, residues of particular interest in one subunit are displayed as van der Waals spheres, and water molecules within a distance of 4.5 Å of these residues are shown as sticks. The solvent accessible crevice near the β 1 strand is displayed in orange (transparent). **C**, Solvent accessible surface area (SASA) of β 1 strand residues and Tyr62 (equivalent to α 1 subunit Phe64) in the GABA_A β 3 homopentamer. The SASA of the homologous residues we chose for functional analysis are (as mean \pm S.D.) 1.34 ± 0.2 nm² for Asn41, 0.05 ± 0.04 nm² for Ile42, 1.85 ± 0.26 nm² for Asp43, 0.07 ± 0.06 nm² for Ile44 and 1.07 ± 0.18 nm² for Ala45 in the β 1 strand. Tyr62 in loop D of the β 2 strand had a SASA of 1.93 ± 0.2 nm².

Figure 2. β 1 strand residues influence the apparent potency of GABA. **A**, Representative examples of whole-cell currents evoked by different concentrations of GABA (indicated) mediated by WT or Cys-substituted α 1 β 2 γ 2 receptors. The bar indicates GABA application. **B**, Concentration-response relationships for WT or Cys-substituted α 1 β 2 γ 2 GABA_A receptors. Current amplitudes were expressed as a percentage of the maximum current amplitude recorded from each cell. Each point represents the mean (\pm S.E.M.) of at least 4 recordings. The sigmoidal curve represents the logistic function fitted to the data points. Mean parameters and statistical comparisons from the logistic function are summarised in Table 1.

Figure 3. α 1(F45C) β 2 γ 2 GABA_A receptors display a biphasic GABA concentration-response relationship and are more spontaneously active. **A**, Concentration-response relationship for α 1(F45C) β 2 γ 2 GABA_A receptors. Current amplitudes were expressed as a percentage of maximum. Each point represents the mean \pm S.E.M. of at least 4 recordings. The solid black line shows the two component logistic function fit to the data points. The two solid grey lines show the separate components of the logistic function fit. The fit parameters are summarised in Table 1. The logistic function fit for WT α 1 β 2 γ 2 receptors is also reproduced here for comparison (grey dotted line). Inset shows representative example of whole-cell currents evoked by different concentrations of GABA (indicated) mediated by α 1(F45C) β 2 γ 2 GABA_A receptors. The bar indicates GABA application. **B**, Representative examples of whole-cell currents evoked by a maximally efficacious concentration of

GABA (grey traces) or inhibition of standing current by PTX (black trace) at $\alpha 1\beta 2\gamma 2$ or $\alpha 1(F45C)\beta 2\gamma 2$ receptors. The black bar indicates GABA or PTX application. The inset shows on an expanded scale, PTX inhibition of standing current. **C**, Bar graph shows mean (\pm S.E.M.) PTX inhibited standing current (I_{PTX}) expressed as a percentage of the total current (I_{total}) for $\alpha 1\beta 2\gamma 2$ ($n = 5$) and $\alpha 1(F45C)\beta 2\gamma 2$ ($n = 10$) GABA_A receptors. The difference in mean PTX component was significantly higher at $\alpha 1(F45C)\beta 2\gamma 2$ receptors (* $P = 0.014$; t-test).

Figure 4. Accessibility of Cys substituted $\beta 1$ strand residues to sulfhydryl modification. **A**, Representative examples of the effect of MTSEA on WT $\alpha 1\beta 2\gamma 2$ and $\alpha 1(F64C)\beta 2\gamma 2$ receptors. MTSEA (10 mM) was applied to a cell expressing WT $\alpha 1\beta 2\gamma 2$ receptors prior to recording a current evoked by the EC₅₀ concentration (10 μ M, black bar) of GABA. Also shown is the effect of MTSEA (2 mM) on $\alpha 1(F64C)\beta 2\gamma 2$ receptors. MTSEA substantially reduced the amplitude of the EC₅₀ (10 mM) GABA-evoked (black bar) current through $\alpha 1(F64C)\beta 2\gamma 2$ receptors. Modification was reversed following application of DTT (10 mM). **B**, Representative examples of MTSEA (2 mM) on $\beta 1$ strand Cys-substituted $\alpha 1\beta 2\gamma 2$ receptors. MTSEA (arrow) reduced the current amplitude of $\alpha 1(D43C)\beta 2\gamma 2$ and $\alpha 1(T47C)\beta 2\gamma 2$ receptors. **C**, Effect of MTSEA (2 mM) on spontaneous activity of $\alpha 1(F45C)\beta 2\gamma 2$ receptors. Spontaneous activity was quantified as I_{PTX}/I_{total} . The level of spontaneous activity in control conditions was $0.51 \pm 0.11\%$ ($n = 10$). In the presence of MTSEA (2 mM), it was $0.38 \pm 0.17\%$ ($n = 5$). The difference was not statistically significant. ($P > 0.05$; t-test). **D**, Bar graph shows mean (\pm S.E.M.) percentage change in current amplitude following MTSEA treatment in WT and mutant $\alpha 1\beta 2\gamma 2$ receptors. The change in GABA-evoked current amplitude was $11 \pm 5.1\%$ ($n = 6$) for WT $\alpha 1\beta 2\gamma 2$, $-46 \pm 2.1\%$ ($n = 8$) for $\alpha 1(D43C)\beta 2\gamma 2$, $21 \pm 7.5\%$ ($n = 3$) for $\alpha 1(I44C)\beta 2\gamma 2$, $5.4 \pm 6.9\%$ ($n = 4$) for $\alpha 1(F45C)\beta 2\gamma 2$, $-1.1 \pm 8.1\%$ ($n = 3$) for $\alpha 1(V46C)\beta 2\gamma 2$, $-49 \pm 5.0\%$ ($n = 15$) for $\alpha 1(T47C)\beta 2\gamma 2$ and $-88 \pm 2.4\%$ ($n = 4$) for $\alpha 1(F64C)\beta 2\gamma 2$ receptors. There was a statistically significant difference in the percentage change between $\alpha 1(D43C)\beta 2\gamma 2$, $\alpha 1(T47C)\beta 2\gamma 2$ and $\alpha 1(F64C)\beta 2\gamma 2$ receptors when compared to WT $\alpha 1\beta 2\gamma 2$ receptors (* $P < 0.0001$; one-way ANOVA; *post-hoc* Dunnet's comparison vs WT).

Figure 5. Rate of MTSEA modification of Cys64, Cys43 and Cys47. **A**, Representative examples of EC₅₀ GABA-evoked currents before and after cumulative MTSEA application at $\alpha 1(F64C)\beta 2\gamma 2$, $\alpha 1(D43C)\beta 2\gamma 2$ and $\alpha 1(T47C)\beta 2\gamma 2$ receptors. The first current in each trace indicates the steady state EC₅₀ GABA-evoked current amplitude in control conditions. MTSEA was applied for the indicated time (arrow) and a second EC₅₀ GABA-evoked current was recorded. Cumulative application of MTSEA led to a progressive reduction in EC₅₀ GABA-evoked current amplitudes. **B**, Rate of MTSEA modification of $\alpha 1(F64C)\beta 2\gamma 2$ ($n = 4$), $\alpha 1(D43C)\beta 2\gamma 2$ ($n = 6$) and $\alpha 1(T47C)\beta 2\gamma 2$ ($n = 8$) receptors. The line shows the exponential function fitted to the data points. **C**, Bar graph shows mean (\pm S.E.M.) τ of

MTSEA modification of $\alpha 1(\text{F64C})\beta 2\gamma 2$ (0.065 ± 0.020 s; $n = 4$), $\alpha 1(\text{D43C})\beta 2\gamma 2$ (0.16 ± 0.020 s; $n = 6$) and $\alpha 1(\text{T47C})\beta 2\gamma 2$ (1.8 ± 0.19 s; $n = 8$) receptors. The τ of MTSEA modification was significantly larger for $\alpha 1(\text{T47C})\beta 2\gamma 2$ receptors compared with $\alpha 1(\text{D43C})\beta 2\gamma 2$ and $\alpha 1(\text{F64C})\beta 2\gamma 2$ receptors (* and # $P < 0.0001$; one-way ANOVA; *post hoc* Tukey's comparison).

Figure 6. Effect of GABA and propofol on MTSEA modification of Cys substituted receptors. **A**, Rate of MTSEA (10 μM) modification of $\alpha 1(\text{F64C})\beta 2\gamma 2$ receptors in control (solid circles), in the presence of GABA (300 mM; open squares) or propofol (10 μM ; open triangles). The line shows the exponential function fitted to the data points. Inset in **A** shows, on an expanded time scale, the difference in rate of MTSEA modification in the presence or absence of agonists. **B**, The bar graph shows mean (\pm S.E.M.) τ MTSEA modification of $\alpha 1(\text{F64C})\beta 2\gamma 2$ receptors. The τ of MTSEA modification in control, GABA and propofol was 0.065 ± 0.02 s ($n = 4$), 5.5 ± 1.3 s ($n = 4$) and 0.057 ± 0.001 s ($n = 5$). The presence of GABA significantly increased the τ of MTSEA modification (* $P < 0.001$; one-way ANOVA; *post hoc* Tukey's comparison). **C**, The bar graph shows mean (\pm S.E.M.) extent of MTSEA modification of $\alpha 1(\text{F64C})\beta 2\gamma 2$ receptors. The extent of MTSEA modification in control, GABA and propofol was $12 \pm 2.4\%$ ($n = 4$), $43 \pm 3.8\%$ ($n = 4$) and $12 \pm 4.4\%$ ($n = 5$). The presence of GABA significantly reduced the extent of MTSEA modification (* $P < 0.0001$; one-way ANOVA; *post hoc* Tukey's comparison). **D**, Same as **A**, the rate of MTSEA (100 μM) modification of $\alpha 1(\text{D43C})\beta 2\gamma 2$ receptors. **E**, Mean (\pm S.E.M.) τ MTSEA modification of $\alpha 1(\text{D43C})\beta 2\gamma 2$ receptors. The τ of MTSEA modification in control, GABA (300 mM) and propofol (10 μM) was 0.16 ± 0.02 s ($n = 6$), 0.41 ± 0.10 s ($n = 6$) and 0.32 ± 0.054 s ($n = 7$). The presence of GABA significantly increased the τ of MTSEA modification (* $P < 0.05$; one-way ANOVA; *post hoc* Tukey's comparison). **F**, Mean (\pm S.E.M.) extent of MTSEA modification of $\alpha 1(\text{D43C})\beta 2\gamma 2$ receptors. The extent of MTSEA modification in control, GABA and propofol was $60 \pm 2.2\%$ ($n = 6$), $71 \pm 3.4\%$ ($n = 6$) and $70 \pm 3.2\%$ ($n = 7$). The presence of GABA and propofol significantly reduced the extent of MTSEA modification (* $P < 0.05$; one-way ANOVA; *post hoc* Tukey's comparison). **G**, Same as **A**, the rate of MTSEA (100 μM) modification of $\alpha 1(\text{T47C})\beta 2\gamma 2$ receptors. **H**, Mean (\pm S.E.M.) τ MTSEA modification of $\alpha 1(\text{T47C})\beta 2\gamma 2$ receptors. The τ of MTSEA modification in control, GABA (30 mM) and propofol (10 μM) was 1.8 ± 0.19 s ($n = 8$), 3.1 ± 0.59 s ($n = 5$) and 2.2 ± 0.54 s ($n = 6$). There was no significant difference in the τ of modification ($P = 0.13$; one-way ANOVA). **I**, Mean (\pm S.E.M.) extent of MTSEA modification of $\alpha 1(\text{T47C})\beta 2\gamma 2$ receptors. The extent of MTSEA modification in control, GABA and propofol was $47 \pm 5.5\%$ ($n = 8$), $69 \pm 4.4\%$ ($n = 5$) and $67 \pm 6.1\%$ ($n = 6$). The presence of GABA and propofol significantly reduced the extent of MTSEA modification (* $P < 0.05$; one-way ANOVA; *post hoc* Tukey's comparison).

Figure 1

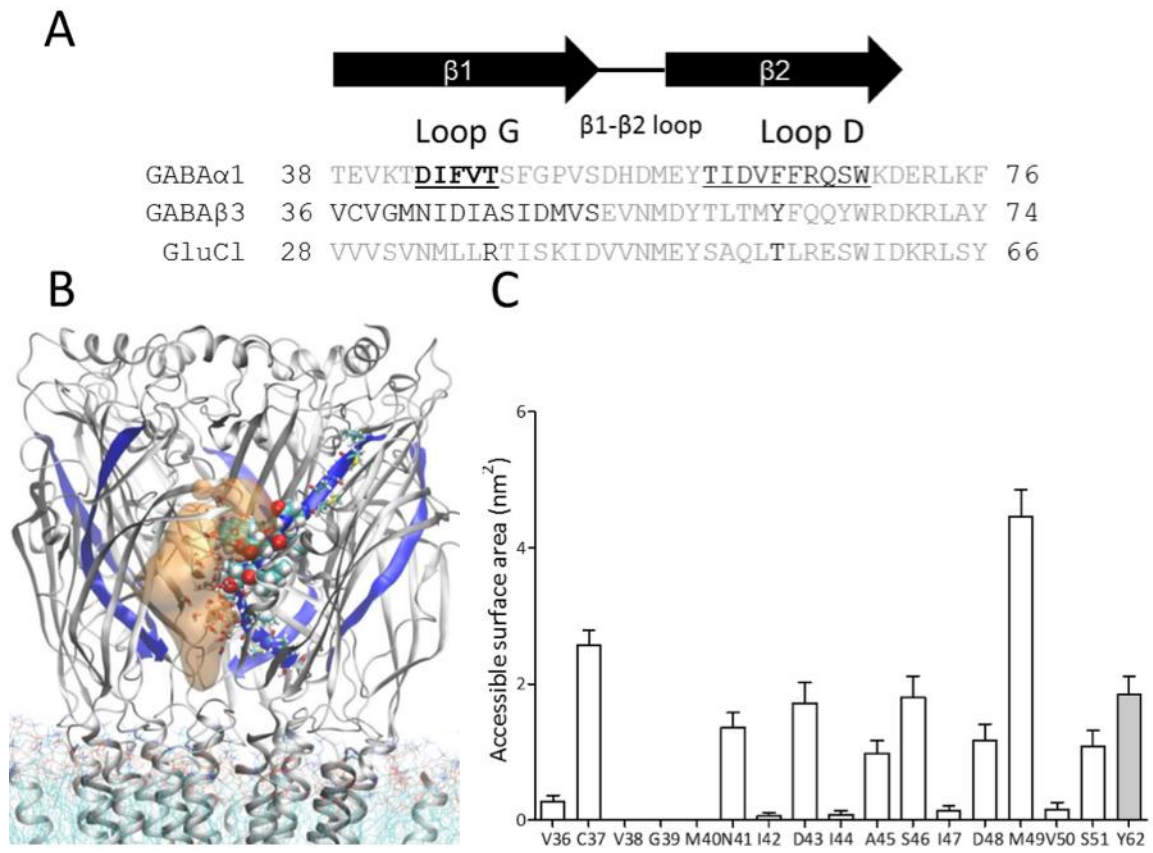


Figure 2

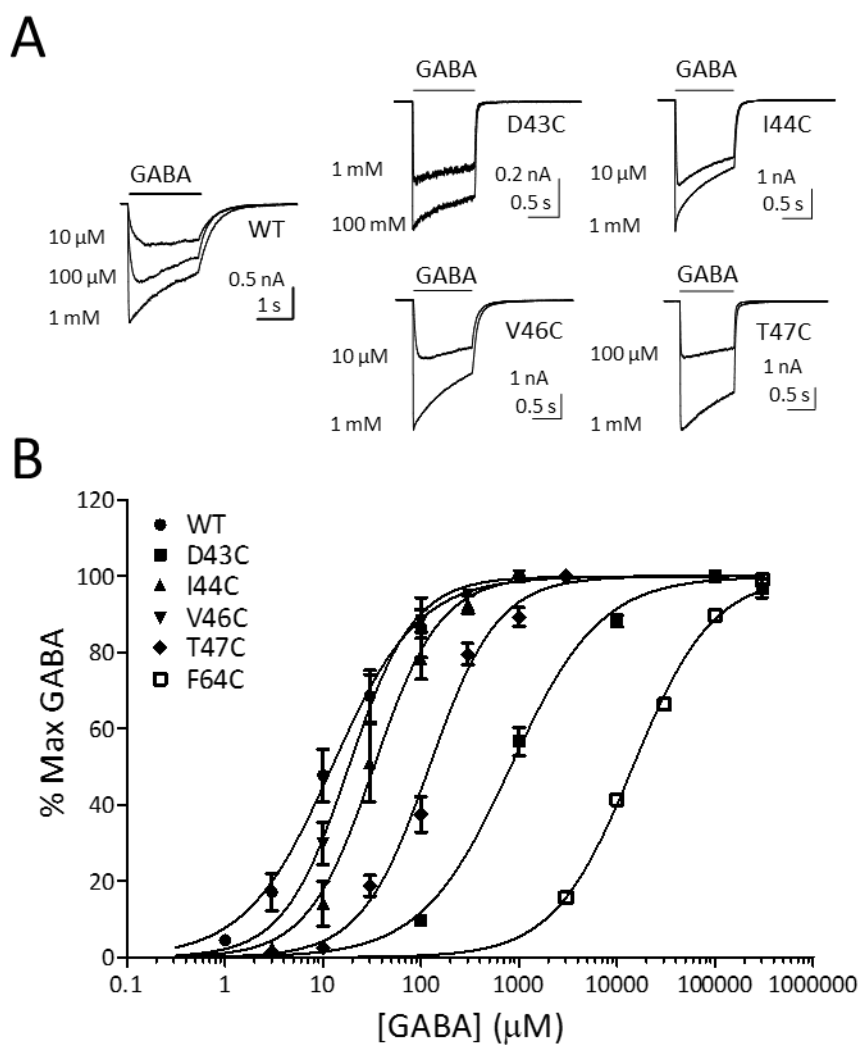


Figure 3

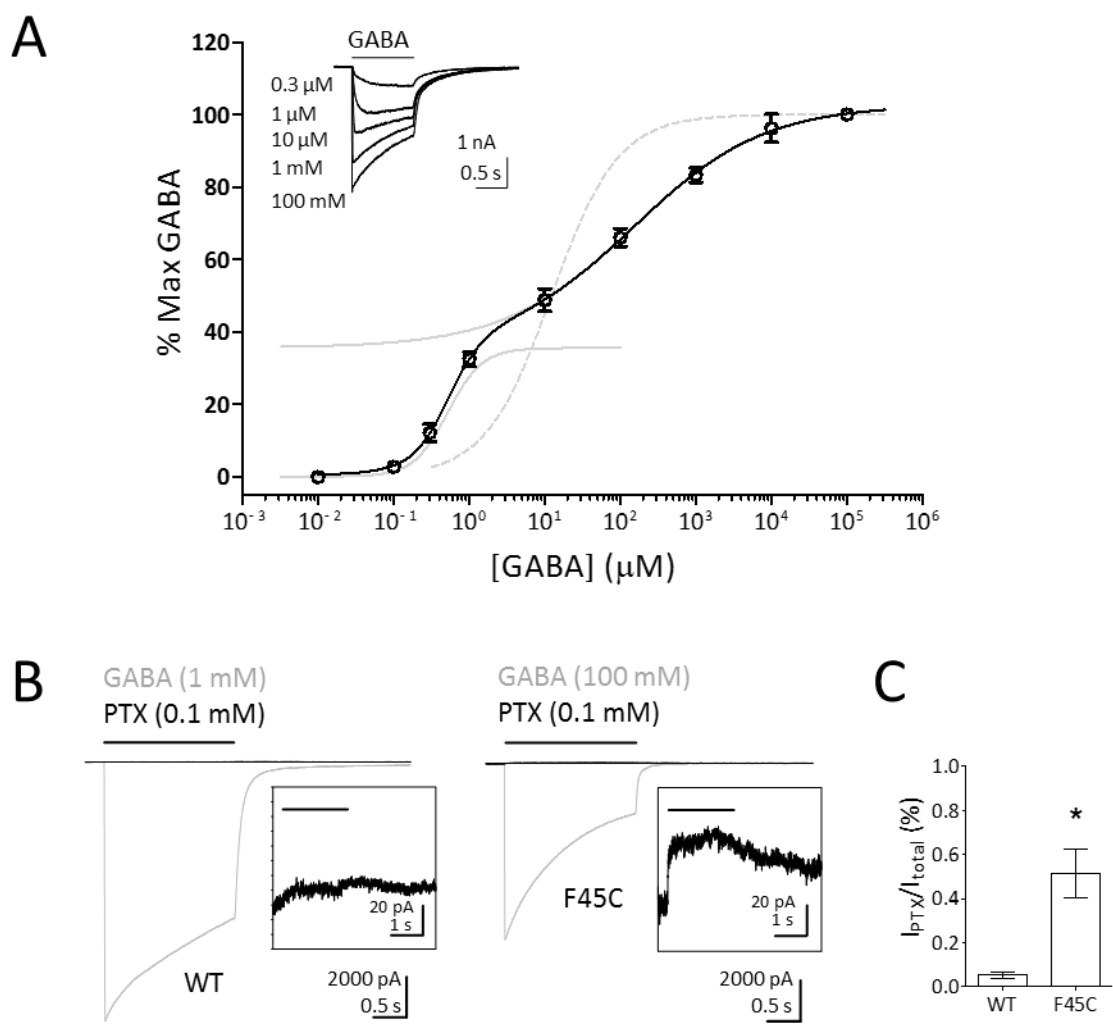


Figure 4

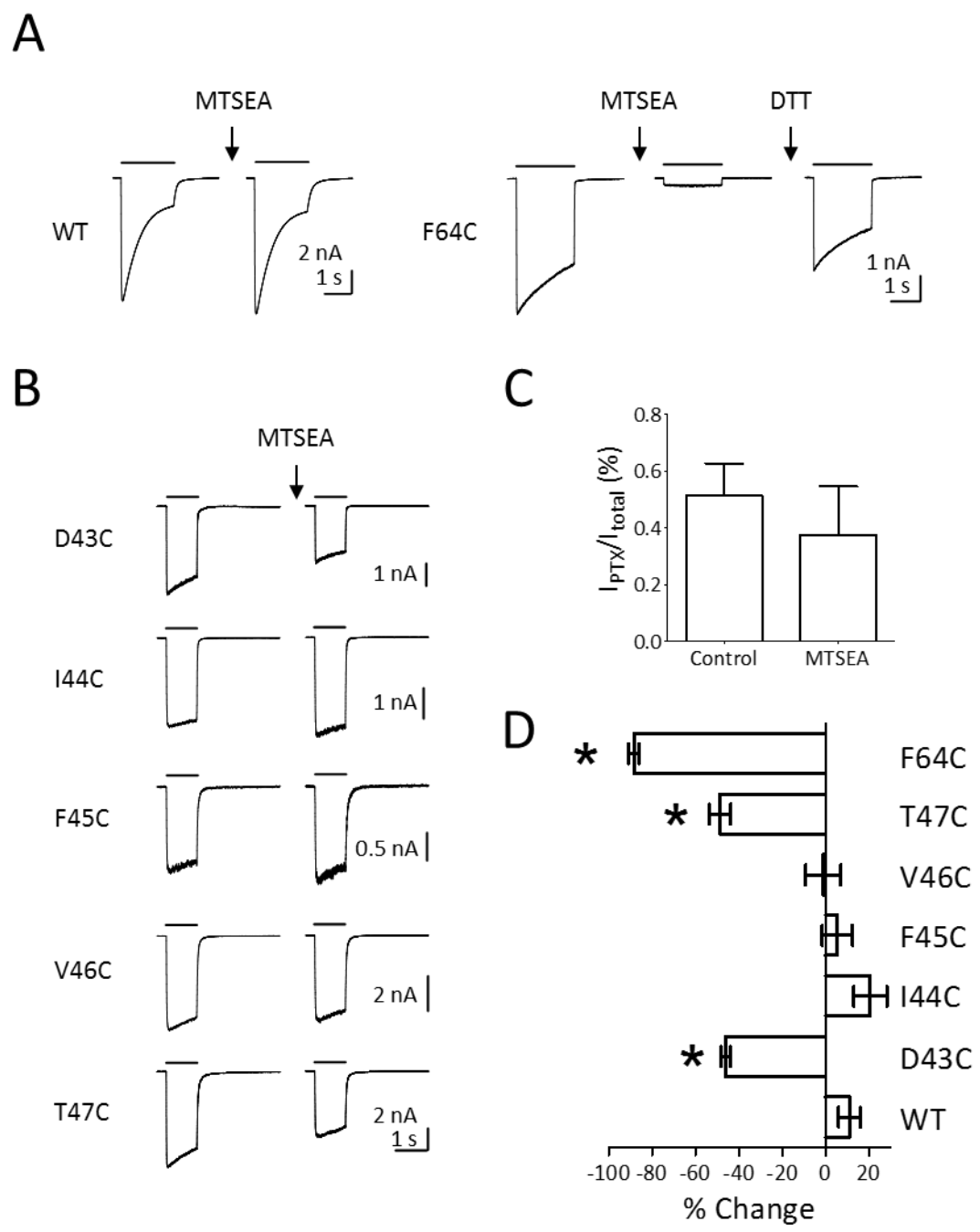


Figure 5

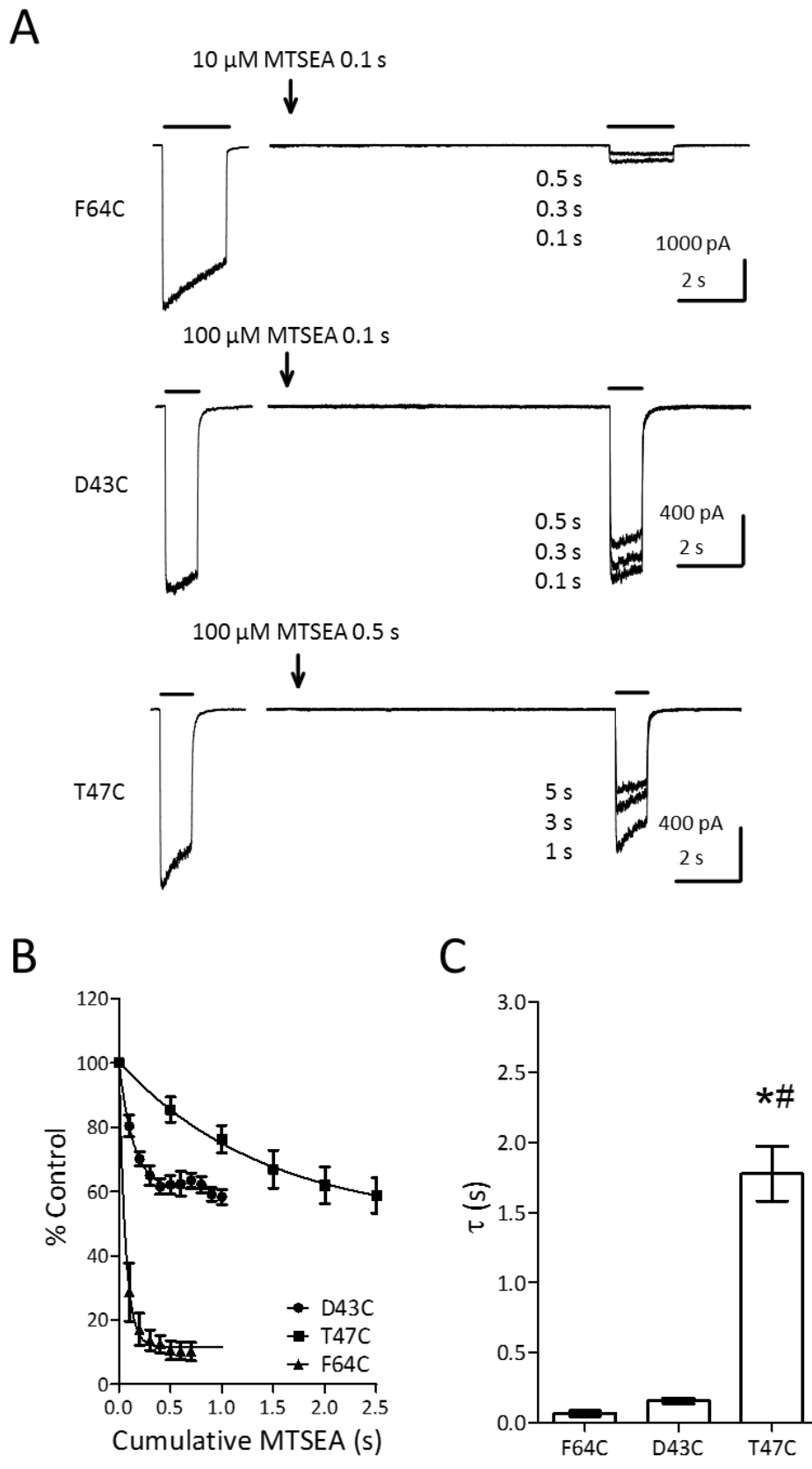


Figure 6

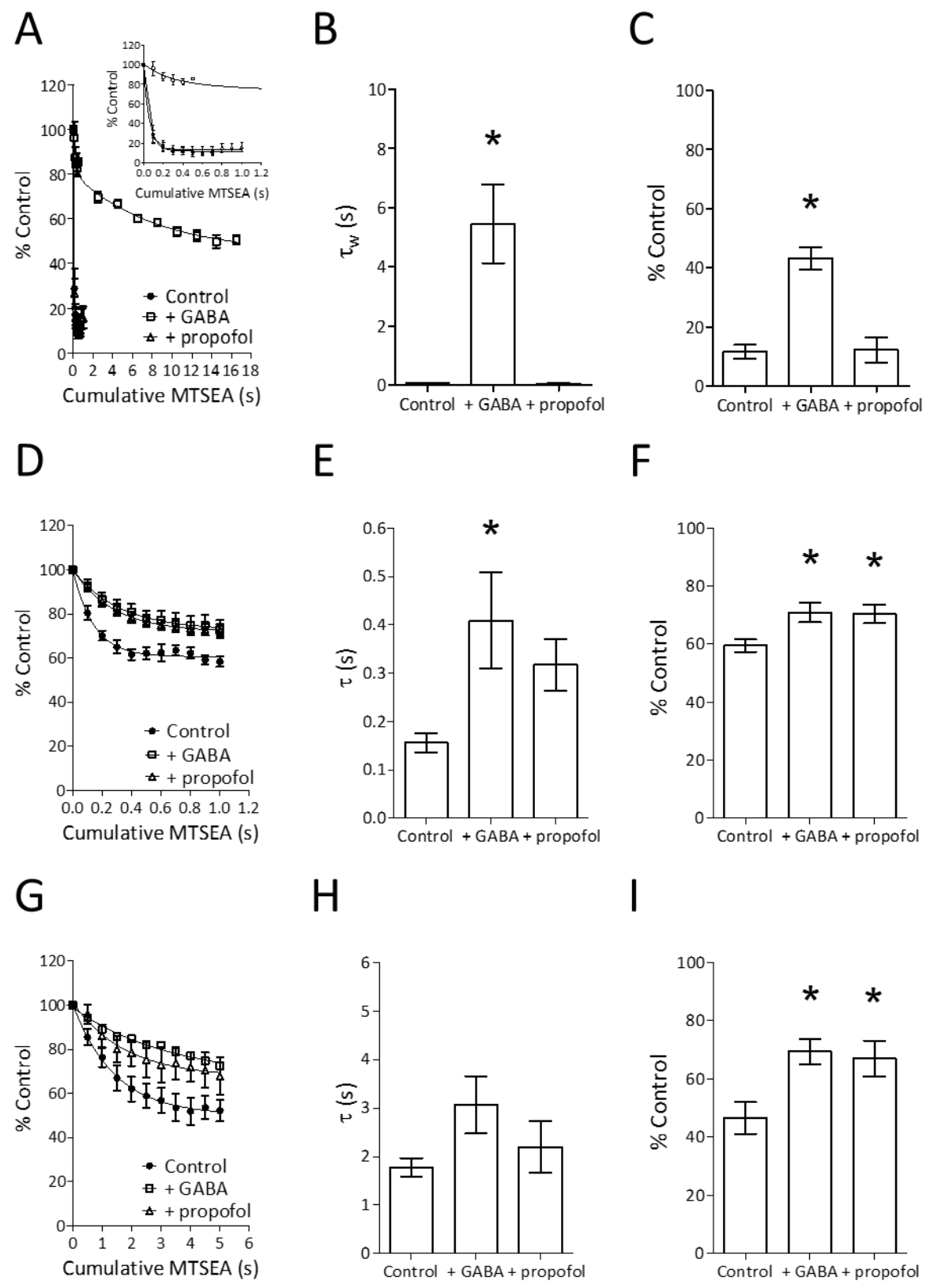


Table 1. Summary of logistic function fit parameters and current density values.

	GABA EC ₅₀ (μM)	Hill slope	Current density (pA pF ⁻¹)
α1β2γ2	17.8 ± 5.1 (6)	1.12 ± 0.17 (6)	1400 ± 250 (20)
α1(D43C)β2γ2	857 ± 161 (5)*	1.01 ± 0.059 (5)	210 ± 69 (8)*
α1(I44C)β2γ2	38.4 ± 11.7 (5)	1.68 ± 0.15 (5)	1200 ± 330 (7)
α1(F45C)β2γ2	HP 0.52 (16) LP 150 (16)	HP 1.91 (16) LP 0.51 (16)	880 ± 130 (27)
α1(V46C)β2γ2	19.0 ± 4.1 (4)	1.48 ± 0.13 (4)	890 ± 230 (7)
α1(T47C)β2γ2	135 ± 13.7 (9)	1.42 ± 0.20 (9)	610 ± 110 (12)*
α1(F64C)β2γ2	19000 ± 1600 (3)†	1.00 ± 0.29 (3)	77.9 ± 33.3 (13)†

Data are provided as mean ± S.E.M. of (n) number of experiments, except EC₅₀ and Hill slope values of α1(F45C)β2γ2 receptors, where the average fit parameters of the high apparent potency (HP) and low apparent potency (LP) components are reported. * Denotes significant difference (P < 0.05) from one-way ANOVA with Dunnet's post hoc comparison with α1β2γ2 values. α1(F45C)β2γ2 receptors were not statistically compared for EC₅₀ and Hill slope values because of the presence of two components in the GABA concentration-response relationship. † Denotes significant difference (P < 0.05) between α1(F64C)β2γ2 receptors and α1β2γ2 receptors from t-test.

Table 2. Summary of second-order rate constants for MTSEA modification.

	MTSEA k_2 ($M^{-1} s^{-1}$)	MTSEA + GABA k_2 ($M^{-1} s^{-1}$)	MTSEA + propofol k_2 ($M^{-1} s^{-1}$)
$\alpha 1(D43C)\beta 2\gamma 2$	70,030 \pm 9,629 (6)	34,530 \pm 10,420 (6)*	38,860 \pm 8,437 (7)
$\alpha 1(T47C)\beta 2\gamma 2$	6,216 \pm 784.8 (8)	3,892 \pm 873.6 (5)	6,049 \pm 1,271 (6)
$\alpha 1(F64C)\beta 2\gamma 2$	1,944,000 \pm 460,500 (4)†	24,660 \pm 9,129 (4)*	1,832,000 \pm 205,400 (5)

Data are provided as mean \pm S.E.M. of (n) number of experiments. The second-order rate constants are calculated by dividing the rate of decay obtained from the exponential fits to data which generated the rate of MTSEA modification data, by the concentration of MTSEA used. The concentration of MTSEA used was 100 μ M for $\alpha 1(D43C)\beta 2\gamma 2$ and $\alpha 1(T47C)\beta 2\gamma 2$ receptors, and 10 μ M for $\alpha 1(F64C)\beta 2\gamma 2$ receptors. * Denotes significant difference ($P < 0.05$) from one-way ANOVA with post hoc Tukey's comparison between the presence or absence of saturating concentrations of GABA, or activating concentrations of propofol. † Denotes significant difference ($P < 0.05$) from one-way ANOVA with post hoc Tukey's comparison between MTSEA k_2 of different mutants.