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Alphaxalone Binds in Inner Transmembrane $\beta^+-\alpha^-$ Interfaces of $\alpha 1\beta 3\gamma 2$ γ -Aminobutyric Acid Type A Receptors

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Abstract

Background—Neurosteroids like alphaxalone are potent anxiolytics, anticonvulsants, amnestics, and sedative-hypnotics, effects linked to enhancement of GABA_A receptor gating in the central nervous system. Data locating neurosteroid binding sites on synaptic $\alpha\beta\gamma$ GABA_A receptors are sparse and inconsistent. Some evidence points to outer transmembrane $\beta^+-\alpha^-$ interfacial pockets, near sites that bind the anesthetics etomidate and propofol. Other evidence suggests that steroids bind more intracellularly in $\beta^+-\alpha^-$ interfaces.

Methods—We created 12 single-residue $\beta 3$ cysteine mutations: $\beta 3T262C$ and $\beta 3T266C$ in $\beta 3$ -M2; and $\beta 3M283C$, $\beta 3Y284C$, $\beta 3M286C$, $\beta 3G287C$, $\beta 3F289C$, $\beta 3V290C$, $\beta 3F293C$, $\beta 3L297C$, $\beta 3E298C$, and $\beta 3F301C$ in $\beta 3$ -M3 helices. We co-expressed $\alpha 1$ and $\gamma 2L$ with each mutant $\beta 3$ subunit in *Xenopus* oocytes and electrophysiologically tested each mutant for covalent sulfhydryl modification by the water soluble reagent para-chloromercuribenzenesulfonate. We then assessed whether receptor-bound alphaxalone, etomidate, or propofol blocked cysteine modification, implying steric hindrance.

Results—Eleven mutant $\beta 3$ subunits, when co-expressed with $\alpha 1$ and $\gamma 2L$, formed functional channels that displayed varied sensitivities to the three anesthetics. Exposure to para-chloromercuribenzenesulfonate produced irreversible functional changes in ten mutant receptors. Protection by alphaxalone was observed in receptors with $\beta 3V290C$, $\beta 3F293C$, $\beta 3L297C$, or $\beta 3F301C$ mutations. Both etomidate and propofol protected receptors with $\beta 3M286C$ or $\beta 3V290C$ mutations. Etomidate also protected $\beta 3F289C$. In $\alpha 1\beta 3\gamma 2L$ structural homology models, all these protected residues are located in transmembrane $\beta^+-\alpha^-$ interfaces.

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Conclusions—Alphaxalone binds in transmembrane $\beta^+-\alpha^-$ pockets of synaptic GABA_A receptors that are adjacent and intracellular to sites for the potent anesthetics etomidate and propofol.

Keywords

Ligand-gated ion channel; glycine receptor; GABA; GluCl; general anesthetic; etomidate; propofol; neurosteroid; neuroactive steroid; cysteine mutation; modification; photolabel; electrophysiology; allosteric modulator; allosteric agonist

Introduction

Neurosteroids (neuroactive steroids), including the general anesthetic alphaxalone (ALX), allopregnanolone, and tetrahydro-deoxycorticosterone, are potent rapid-acting anxiolytics, anticonvulsants, amnestics, and sedative-hypnotics¹. These effects are linked to enhanced gating of γ -aminobutyric acid type A (GABA_A) receptors, the main inhibitory neurotransmitter receptors in mammalian brain and major molecular targets for the general anesthetics propofol (PRO) and etomidate (ETO)^{2,3}. Typical synaptic GABA_A receptors consist of 2 α , 2 β , and 1 γ subunits arranged $\beta\alpha\beta\alpha\gamma$ counterclockwise, viewed from the extracellular space⁴. Each GABA_A subunit contains an N-terminal extracellular domain and a transmembrane domain with four alpha helices: M1 to M4. Five M2 helices surround a receptor's central chloride channel, while M1 and M3 helices form an intermediate ring between M2s and M4 helices. Subunit interfaces are designated $\beta^+-\alpha^-$ (two per receptor), $\alpha^+-\beta^-$, $\gamma^+-\beta^-$, and $\alpha^+-\gamma^-$, where '+' corresponds to the M3 face and '-' is the M1 face.

Data locating neurosteroid sites on GABA_A receptors are sparse and inconsistent (Table 1⁵⁻²²). Pharmacokinetic studies indicate that neurosteroids reach GABA_A receptors *via* membrane lipids²³. Mutations in $\alpha 1$ -M1 at $\alpha 1$ M236, $\alpha 1$ T237, and $\alpha 1$ I239 reduce neurosteroid sensitivity^{5,13}. These residues map to outer transmembrane $\beta^+-\alpha^-$ clefts in homology models based on glutamate-gated chloride (GluCl) channels from *Caenorhabditis elegans*²⁴ (Fig 1²⁵) and are identified by photolabeling and substituted cysteine modification-protection (SCAMP) studies as contacts for ETO and PRO (Table 1)²⁶. Ivermectin binds to outer transmembrane inter-subunit pockets on GluCl²⁴ and triiodothyronine displaces both ivermectin and allopregnanolone from homologous GABA_A receptor sites, including the ETO/PRO sites²⁷. Thus, neurosteroids may act through the outer transmembrane $\beta^+-\alpha^-$ pockets where ETO and PRO bind.

Other evidence indicates that neurosteroid sites are separate from ETO and PRO sites. Neurosteroids synergize with ETO and its derivatives when co-applied to GABA_A receptors^{28,29}. Previous SCAMP experiments find no ALX interactions at several ETO and PRO contacts in outer transmembrane $\beta^+-\alpha^-$ clefts or other homologous pockets in $\alpha 1\beta 3\gamma 2L$ receptors^{5,21}. Other evidence points to inner transmembrane $\beta^+-\alpha^-$ neurosteroid sites. Mutations in inner $\alpha 1$ -M1 at $\alpha 1$ Q242 reduce neurosteroid sensitivity^{13,14}. The photolabel (3 $\alpha,5\beta$)-6-azi-pregnanolone (6-AziP) incorporates in inner $\beta 3$ -M3 at $\beta 3$ F301, but this study used $\beta 3$ homomeric receptors²². Finally, $\beta 2$ Y284 mutations also impair neurosteroid effects¹³. This residue's location in $\beta 3$ crystals³⁰ and homology models (Fig 1) suggests neurosteroid sites within $\beta 3$ intra-subunit helix bundles.

To test whether ALX binds in $\beta^+ - \alpha^-$ transmembrane clefts and compare ALX sites to those for ETO and PRO, we used SCAMP to assess drug contacts on β_3 -M2 and β_3 -M3 helices in $\alpha_1\beta_3\gamma_2L$ receptors. Using the structure of β_3 homomeric receptors³⁰ and our GluCl-based structural homology model²⁵ (Fig 1), we selected residues spanning most of the β_3 -M3 helix, from β_3 M283 (outer) to β_3 F301 (inner), most facing the $\beta^+ - \alpha^-$ interface and several facing the intra-subunit β_3 helix pocket. Our results suggest that ALX contacts β_3 -M3 at $\beta^+ - \alpha^-$ interfacial residues that are adjacent and intracellular to those for PRO and ETO.

Materials and Methods

Animals

Oocytes were harvested from female *Xenopus laevis* frogs in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal use in this study was approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee (protocol #2005N000051). Frogs were housed and maintained in a veterinarian-supervised facility and anesthetized in tricaine during oocyte collection. All efforts were made to minimize suffering.

Materials

Alphaxalone was purchased from Tocris Bioscience (Bristol, UK) and propofol (2,6-diisopropylphenol) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Both were stored as 10 mM solutions in DMSO and diluted in electrophysiology buffer for experiments. R-Etomidate was purchased from Hospira, Inc (Lake Forest, IL, USA) as a 2 mg/ml (~8.2 mM) solution in 35% propylene glycol:water and diluted in electrophysiology buffer for experiments. We have previously shown that DMSO and propylene glycol at the dilutions used during electrophysiology experiments produce no effects on GABA_A receptor function²⁵. R-mTFD-MPAB (R-allyl-m-trifluoromethyl-mephobarbital)³¹ was a gift from Prof. Karol Bruzik, PhD (Dept. of Medicinal Chemistry and Pharmacognosy, University of Illinois, Chicago, USA). It was stored as a 100 mM solution in DMSO and diluted in electrophysiology buffer for experiments. Para-chloromercuribenzenesulfonic acid sodium salt (pCMBS) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Fresh pCMBS stock solutions in electrophysiology buffer were prepared on the day of use, and kept on ice until final dilution. γ -Aminobutyric acid (GABA), picrotoxin, salts, and buffers were purchased from Sigma-Aldrich.

GABA_A Receptor Expression in *Xenopus* Oocytes

Oocytes were prepared for use as previously described⁵. Complementary DNAs encoding human α_1 , β_3 , and γ_2L GABA_A receptor subunits in pCDNA3.1 expression vectors (Thermo Fisher Scientific, Waltham, MA, USA) were used. Cysteine mutations were introduced into β_3 by site-directed mutagenesis using QuikChange kits (Agilent Technologies, Santa Clara, CA, USA). After sequencing several clones through the entire coding region, one clone for each mutant was chosen for further use. Messenger RNAs were synthesized on linearized DNA templates using mMessage mMachine kits (Thermo Fisher), purified, and combined at ratios of 1 α_1 :1 β_3 :5 γ (final concentration 1 ng/nl in RNAase-free water). Oocytes were injected with ~50 ng mRNA mix and incubated in ND96 buffer (in

mM: 96 NaCl, 2 KCl, 1 CaCl₂, 0.8 MgCl₂, 10 HEPES, pH 7.5) supplemented with ciprofloxacin (2 mg/ml) and amikacin (100 µg/ml) at 17 °C for 48 to 72 hours before electrophysiological studies.

Two Electrode Voltage-Clamp Electrophysiology

Electrophysiological experiments were performed in ND96 buffer at 21 to 23 °C as previously described⁵. Oocytes were placed in a 30 µl custom flow-cell, impaled with borosilicate glass micro-electrodes filled with 3 M KCl (resistance < 1 MΩ), then voltage-clamped at -50 mV (model OC-725C, Warner Instruments, Hamden CT, USA). Superfusion solutions in ND96 were controlled by electrical valves (VC-8, Warner Instruments) and delivered at a rate of 2–3 ml/min from glass reservoir syringes *via* PTFE tubing and a PTFE micro-manifold (MP-8, Warner Instruments). Specialized software and a digital input/output interface (pClamp 8.0 and Digidata 1322, both from Molecular Devices, Sunnyvale, CA) were used to coordinate delivery of solutions and recordings. Current signals were filtered at 1 kHz, digitized at 100 Hz, and stored on a computer disk for offline analysis.

GABA concentration-responses, spontaneous receptor activity, and GABA efficacy

Each mutant receptor was initially characterized to establish its sensitivity to GABA, maximal GABA efficacy, and whether it was spontaneously active. Voltage-clamped oocytes were exposed to GABA solutions (range 0.1 µM to 10 mM) for 10 to 20 s, followed by 5 minute ND96 wash. Normalization sweeps at the maximum GABA concentration for the specific receptor (>10 x EC₅₀; 1–10 mM) were recorded every second or third experiment. At least 3 oocytes from two different frogs were used for each concentration-response.

Spontaneous activation of GABA_A receptors (in the absence of GABA or anesthetics) was assessed by applying 2 mM picrotoxin to voltage-clamped oocytes. Reversible outward currents during picrotoxin application represent closure of spontaneously active channels. Spontaneous activity was normalized to maximal GABA-elicited current in the same cell (n = 3 cells).

Maximal GABA efficacy for each receptor was estimated by comparing peak currents elicited with maximal GABA (1–10 mM) to currents elicited with high GABA supplemented with either 2.5 to 5 µM ALX or 3.2 to 6.4 µM ETO, depending on the receptor's drug sensitivity (see below). Agonist efficacy was calculated by normalizing maximal GABA responses to GABA + anesthetic responses in the same cell, assuming the latter represents 100% activation (n = 3 cells).

GABA EC5 enhancement

Each mutant was also characterized for sensitivity to ETO, PRO, and ALX. Voltage-clamped oocytes expressing GABA_A receptors were repetitively exposed for 20 s to GABA EC5 (eliciting ~ 5% of maximal GABA response) separated by 5 min wash until three stable responses (varying by less than 5%) were sequentially recorded. The oocyte was then exposed for 30 s to anesthetic, followed by 20 s exposure to a solution containing GABA EC5 combined with anesthetic at 2 x EC50 for loss-of-righting-reflexes (LoRR) in tadpoles: 2.5 µM alphaxalone³², 3.2 µM etomidate³³, or 5 µM propofol³⁴. For each receptor type

and three anesthetics, multiple measurements of current response to GABA EC5 and GABA EC5 + anesthetic were obtained in at least four oocytes from two different frogs. EC5 enhancement (mean \pm sem; n = 4) was calculated from the set of individual oocyte ratios of currents measured with anesthetic present to EC5 GABA alone.

Substituted Cysteine Modification and Protection (SCAMP)

SCAMP studies followed the approach we have described previously^{5,21}. In each mutant receptor, functional effects and rates of cysteine modification were assessed electrophysiologically after applications of pCMBS either alone or together with maximally activating GABA (1 to 10 mM). Before and after pCMBS exposures, voltage clamped *Xenopus* oocytes expressing mutant receptors were exposed to first GABA EC5 (low) and then a maximally activating GABA concentration (high; 1–10 mM). After 5 minute wash, oocytes were exposed for 10 to 20 s to pCMBS (1 μ M to 1 mM), a water-soluble sulfhydryl modifying reagent, either alone or co-applied with maximal GABA (1 to 10 mM). PCMBS exposure was followed by a 3–5 minute wash in ND96. Electrophysiological responses to low and high GABA were then re-tested to assess any irreversible changes in receptor function produced by pCMBS modification (in most cases an increase in the ratio of low versus high GABA-induced peak currents). By testing a range of pCMBS concentrations this way, we identified conditions resulting in maximal modification effects and those appropriate for studying modification rates.

To measure apparent modification rates, pCMBS exposure conditions (concentration x time) were chosen that produced about 10% of the maximal modification effect per cycle. In nearly all mutants, higher pCMBS concentrations were needed to irreversibly affect receptors when applied alone than when co-applied with GABA. Voltage-clamped oocytes were first repeatedly tested for responses to both low and high GABA, then washed for 5 min in ND96, to confirm that the response ratio was stable (< 5% variation) before pCMBS exposure. Oocytes were then exposed for 5 to 10 s to pCMBS (with or without GABA), followed by 5 minute wash, and re-testing for low and high GABA responses. At least three cycles of pCMBS exposure/wash/low:high GABA response testing were performed on each oocyte used for rate analysis. The series of modification cycles under the selected conditions typically produced less than 50% of the maximal modification effect. A final modification cycle was performed using 10 x pCMBS concentration for 20 s to fully modify receptors, and subsequent electrophysiological response was assessed as the maximal modification effect.

Protection experiments were performed in the presence of maximally activating GABA, as previously described⁵, so control modification conditions were pCMBS + GABA. Oocytes were exposed to anesthetic for 30 s followed by exposure to a solution of pCMBS + GABA + anesthetic. Post-modification wash and response tests were identical to control modification conditions (i.e. usually with no anesthetic present, but see below). Anesthetic concentrations used in initial protection studies were chosen to maximize site occupancy, while enabling washout within 5 minutes (10 μ M ETO; 20 μ M PRO; and 10 μ M ALX). In receptors with β 3F289, β 3F293C, and β 3L297C mutations, higher concentrations of anesthetics (50 μ M ETO, 100 μ M PRO, or 50 μ M ALX) were also used in protection

experiments. Under these conditions, anesthetic washout between pCMBS exposure and testing for modification effects was extremely slow. We therefore used an alternative approach to low GABA responses, measuring direct activation by anesthetics alone (50 μ M ETO, 100 μ M PRO, or 50 μ M ALX), normalized to high GABA responses. At least two anesthetics were tested in the same manner, to test for drug-specific interactions. In the case of receptors with β 3V290C mutations, we tested for allosteric effects (i.e. whether all anesthetics similarly affect pCMBS modification), by including SCAMP studies with 10 μ M mTFD-MPAB, a barbiturate hypnotic that acts through GABA_A receptor sites outside the β ⁺- α ⁻ interfaces^{8,31}. For each cysteine mutant, at least 5 oocytes were studied in control modification experiments and at least 4 oocytes were studied in each set of anesthetic protection experiments. Group sample sizes of 5 per group were based both on prior experience and a power analysis performed as previously described⁵, using a one-tail t-test with $\alpha = 0.017$ (adjusted for three drug comparisons to each control).

Data analysis and statistics

Results in text and figures are mean \pm sem unless otherwise indicated.

GABA concentration-responses—Digitized GABA concentration-response data was corrected for baseline leak currents and digitally filtered (10 Hz low-pass, Bessel function) using Clampfit 9.0 software (Molecular Devices). Peak currents were normalized to control (maximal currents), and combined GABA data from multiple cells ($n = 3$) was fitted with logistic equations using Prism 5.02 (GraphPad Software Inc, La Jolla, CA):

$$I_{norm} = (I_{max} - I_{min}) / (1 + 10^{(LogEC_{50} - Log[GABA] \times nH)}) + I_{min} \quad \text{Eq. 1}$$

where EC₅₀ is the half-maximal activating GABA concentration, and nH is the Hill slope. Mean GABA EC₅₀ and 95% confidence interval are reported. To assess whether mutations altered GABA EC₅₀ relative to wild-type, we performed sum-of-squares F-tests in Graphpad Prism 5.02, using $p < 0.0045$ as a statistical significance threshold (the Bonferroni correction for $p < 0.05$ with 11 comparisons).

Functional characteristics of mutant receptors—To test whether mutations altered spontaneous activity and/or GABA efficacy from wild-type values we used one-way ANOVA with post-hoc Dunnett's tests (in Prism 5.02). To test whether mutations affected receptor sensitivities to ETO, PRO, or ALX, EC₅ enhancement data for the three equi-potent anesthetic concentrations in wild-type and all functional cysteine mutants was tabulated and analyzed with two-way ANOVA and Bonferroni posttests for wild-type vs. mutation for each anesthetic (Prism 5.02).

SCAMP—Inferences regarding contact between receptor-bound anesthetics and substituted cysteine sidechains were made when an anesthetic inhibited pCMBS modification selectively, with at least one other anesthetic failing to inhibit modification. Apparent pCMBS modification rates were calculated from data for individual oocytes expressing cysteine mutants. Either normalized maximal GABA responses (for α 1 β 3T262C γ 2L) or

normalized low:high GABA response ratios (all other mutants) were plotted against cumulative pCMBS exposure ($M \times s$) and fitted by linear least squares with y-axis intercepts fixed at 1.0. The linear slope, under conditions of partial modification, is presumed to be proportional to the bimolecular reaction rate between pCMBS and the substituted cysteine sulfhydryl.

For $\alpha 1\beta 3\gamma 2L$ data, apparent modification rates were calculated as the absolute values of the negative fitted slopes. Absolute slopes less than $10 M^{-1}s^{-1}$ (the lower limit of detection) were assigned a rate of $10 M^{-1}s^{-1}$ for statistical analysis. To identify anesthetics that either accelerated or inhibited modification of each substituted cysteine, apparent rates from control and anesthetic protection studies for that mutant were log transformed, tabulated, and compared using one-way ANOVA (Prism 5.02) with $p < 0.05$ as a significance threshold.

Results

Functional characteristics of $\beta 3$ cysteine mutants

Based on both crystallographic data for $\beta 3$ homomeric GABA_A receptors (PDB 4COF)³⁰ and our $\alpha 1\beta 3\gamma 2L$ structural homology model based on GluCl bound to ivermectin (PDB 3RHW; Fig 1)^{25,26}, we identified nine $\beta 3$ -M2 and M3 helix residues facing the $\beta^+ - \alpha^-$ cleft: T262, T266, M283, M286, F289, V290, F293, L297, and F301. We created mutant $\beta 3$ cDNAs encoding cysteine substitutions at these positions, as well as at Y284, G287, and E298, which are predicted to instead face the intra-subunit $\beta 3$ helix-bundle pocket. Wild-type and mutant $\beta 3$ subunits were co-expressed with wild-type $\alpha 1$ and $\gamma 2L$ subunits in *Xenopus* oocytes and functionally characterized using two-microelectrode voltage clamp electrophysiology. No GABA-activated currents were detected when $\beta 3$ subunits with Y284C mutations were co-expressed with $\alpha 1$ and $\gamma 2L$, consistent with a prior report³⁵. All the other mutations produced GABA-sensitive ion channels with sufficient oocyte currents elicited by 1–10 mM GABA ($\sim 0.5 \mu A$ at -50 mV) for further experiments. Table 2 summarizes GABA EC₅₀, spontaneous activation, apparent maximal GABA efficacy, and the effect of pCMBS application in these mutant receptors, in comparison to wild-type $\alpha 1\beta 3\gamma 2L$. Six mutations ($\beta 3T266C$, $\beta 3M286C$, $\beta 3G287C$, $\beta 3F293C$, $\beta 3L297C$, and $\beta 3E298C$) significantly increased GABA EC₅₀ and one ($\beta 3F289C$) reduced GABA EC₅₀ approximately five-fold. Four mutant receptors characterized by increased GABA EC₅₀ also exhibited significantly reduced GABA efficacy ($\beta 3M286C$, $\beta 3F293C$, $\beta 3L297C$, and $\beta 3E298C$). Like other mutations that sensitize receptors to GABA^{7,36}, $\beta 3F289C$ was associated with both high GABA efficacy and measurable spontaneous activation. Our observations were also consistent with previous studies of $\beta 2M286C$, $\beta 2G287C$, and $\beta 2F289C$ mutations^{20,21,35,37,38}.

Anesthetic sensitivities of cysteine mutants

GABA_A receptor mutations may alter anesthetic modulation, which can in turn affect the conditions appropriate for SCAMP tests for drug contacts. We therefore characterized each mutant receptor's sensitivity to ETO, PRO, and ALX by measuring anesthetic enhancement of activation by EC₅ GABA. Results are summarized in Fig. 2. Drug solutions of 3.2 μM

ETO, 5 μM PRO, and 2.5 μM ALX are all twice the EC₅₀ for tadpole loss-of-righting reflexes and also similarly enhance the gating of wild-type $\alpha 1\beta 3\gamma 2\text{L}$ GABA_A receptors activated with EC₅ GABA⁵ (Fig 2). Compared to wild-type, two mutations, $\beta 3\text{M}286\text{C}$ and $\beta 3\text{F}289\text{C}$ reduced EC₅ enhancement by 3.2 μM ETO, while $\beta 3\text{F}293\text{C}$, $\beta 3\text{L}297\text{C}$, and $\beta 3\text{E}298\text{C}$ increased EC₅ enhancement by ETO. EC₅ enhancement by 5 μM PRO was also reduced by $\beta 3\text{M}286\text{C}$ and $\beta 3\text{F}289\text{C}$, as well as by $\beta 3\text{F}293\text{C}$. EC₅ enhancement by 2.5 μM ALX was reduced by $\beta 3\text{F}289\text{C}$, $\beta 3\text{F}293\text{C}$, and $\beta 3\text{L}297\text{C}$.

Effects of pCMBS on cysteine mutant function

To establish conditions for SCAMP experiments, we examined the effects of pCMBS exposure, both alone and co-applied with GABA, in each of the cysteine mutants. Wild-type $\alpha 1\beta 3\gamma 2\text{L}$ receptors were unaffected by pCMBS exposure at 1 mM for 60 s ($n = 4$). In all but one ($\beta 3\text{M}283\text{C}$) of the functional cysteine-substituted mutant receptors we studied, exposure to pCMBS alone or with maximally-activating GABA concentrations induced consistent irreversible functional changes that significantly differed from repeated baseline GABA responses prior to pCMBS exposure (Fig 3A–I; Table 2). In $\alpha 1\beta 3\text{T}262\text{C}\gamma 2\text{L}$ receptors, pCMBS exposure similarly reduced activation by both low and high GABA (Fig 3A). In the other mutant receptors, pCMBS exposure enhanced GABA sensitivity, increasing low:high response ratios in the range of 2-fold to 13-fold (Table 2). With the exception of $\beta 3\text{G}287\text{C}$, modification in the presence of GABA required lower pCMBS concentrations than without GABA at all substituted cysteines, resulting in faster apparent modification rates (Fig 3J). Results in $\alpha 1\beta 3\text{M}286\text{C}\gamma 2\text{L}$ receptors (currents not shown in Fig 3) were consistent with earlier studies of $\alpha 1\beta 2\text{M}286\text{C}\gamma 2\text{L}$ ^{20,21}.

Anesthetic protection (SCAMP) with ETO, PRO, and ALX

We previously have shown that SCAMP reliably identifies anesthetic contacts when drugs significantly and selectively inhibit pCMBS modification⁵. Thus, apparent initial rates of cysteine modification in control conditions (pCMBS + GABA) were compared to rates in the presence of added ALX, ETO, or PRO in each of the modifiable mutant receptors. We chose control pCMBS modification conditions in the presence of maximally activating GABA because, a) GABA enhances anesthetic binding and thus site occupancy, b) GABA accelerates pCMBS modification (Fig 3J), and c) GABA helps to establish similar mixtures of functional receptor states in both control modification and protection experiments^{6,21}. Initial protection conditions included 10 μM ETO, 20 μM PRO, or 10 μM ALX along with GABA and pCMBS. In some mutant receptors that displayed low apparent affinity for anesthetics, we also used five-fold higher protecting anesthetic concentrations. In these cases, we used equivalent high concentrations of at least one other anesthetic to test for drug-specific protection.

Normalized modification data and rate analyses for nine mutations are shown in Fig 4 and summarized in Fig 4J²¹. The apparent rate of modification of $\alpha 1\beta 3\text{T}262\text{C}\gamma 2\text{L}$ receptors (Fig 4A) was unaffected by ETO (red symbols and lines), but accelerated by PRO (green symbols and lines). Modification of $\alpha 1\beta 3\text{T}266\text{C}\gamma 2\text{L}$ receptors (Fig 4B) was accelerated by all three anesthetics, suggesting an allosteric effect. $\beta 3\text{M}286\text{C}$ protection was fully consistent with previous SCAMP studies of $\alpha 1\beta 2\text{M}286\text{C}\gamma 2\text{L}$ receptors, showing that both

ETO and PRO block modification, while ALX weakly accelerates pCMBS modification (summarized in Fig 4J)^{20,21}. Modification of $\alpha 1\beta 3G287C\gamma 2L$ receptors (Fig 4C) was unaffected by the three anesthetics. Modification of $\alpha 1\beta 3F289C\gamma 2L$ receptors was weakly blocked by 10 μM ETO, unaffected by 20 μM PRO, and accelerated by 10 μM ALX (data not shown). Because this mutant was insensitive to anesthetics (Fig 2), we also tested 50 μM ETO, which inhibited the apparent rate of $\beta 3F289C$ modification over ten-fold, while neither 100 μM PRO nor 50 μM ALX inhibited modification (Fig 4D). Modification of $\alpha 1\beta 3V290C\gamma 2L$ receptors (Fig 4E) was strongly blocked by 10 μM ETO, 20 μM PRO, and 10 μM ALX. To test whether $\beta 3V290C$ modification was allosterically inhibited by anesthetics that do not bind in $\beta^+-\alpha^-$ sites, we also tested the effect of 10 μM mTFD-MPAB, a potent barbiturate that selectively binds to GABA_A receptor $\alpha^+-\beta^-$ and $\gamma^+-\beta^-$ transmembrane interfaces⁸. Modification of receptors with $\beta 3V290C$ mutations was unaffected by 8 μM mTFD-MPAB (Fig 4J), indicating that inhibition of modification by ETO, PRO, and ALX was likely steric rather than allosteric.

Modification of $\alpha 1\beta 3F293C\gamma 2L$ receptors was accelerated by ETO and PRO, but unaffected by 10 μM ALX. Increasing ALX to 20 μM (Fig 4F, dashed purple lines) or 50 μM (solid purple lines) resulted in significantly reduced rates of $\beta 3F293C$ modification in comparison to 50 μM ETO and 100 μM PRO (Fig 4F). Modification of $\alpha 1\beta 3L297C\gamma 2L$ receptors was unaffected by low concentrations of ETO, PRO or ALX (not shown). Because $\alpha 1\beta 3L297C\gamma 2L$ is relatively insensitive to ALX (Fig 2), we performed additional SCAMP experiments with 50 μM ALX vs. 50 μM ETO in this mutant, revealing inhibition by ALX, but not ETO (Fig 4G). Modification of $\alpha 1\beta 3E298C\gamma 2L$ receptors (Fig 4H) was unaffected by any of the anesthetics. Modification of $\alpha 1\beta 3F301C\gamma 2L$ receptors (Fig 4I) was weakly but significantly blocked by 10 to 20 μM ALX and unaffected by 10 to 20 μM ETO.

On the opposite face of the transmembrane $\beta^+-\alpha^-$ cleft, Hosie et al¹³ identified mutant effects on neurosteroid sensitivity at three residues in $\alpha 1$ -M1: $\alpha 1T237$, $\alpha 1I239$, and $\alpha 1Q242$ (Table 1). We have previously reported that receptors with both $\alpha 1I239C$ and $\alpha 1Q242C$ mutations are unaffected by pCMBS, precluding SCAMP studies⁶. To supplement our studies of $\beta 3$ -M2 and $\beta 3$ -M3 residues, we used SCAMP to test whether ALX protects the cysteine substitution at $\alpha 1T237$. No inhibition of pCMBS modification rates in $\alpha 1T237C\beta 3\gamma 2L$ receptors by 10 μM ALX was observed (data not shown), whereas 10 μM ETO inhibited modification, in agreement with previous results⁶.

Discussion

Major Findings

Our aims in this study were to assess hypothesized ALX contacts with $\beta 3$ sidechains that face transmembrane $\beta^+-\alpha^-$ clefts in $\alpha 1\beta 3\gamma 2L$ GABA_A receptors, and to compare these with ETO and PRO contacts. Using electrophysiology, we studied ten mutant receptors with single cysteine-substitutions in $\beta 3$ -M2 or $\beta 3$ -M3 helices, in which the sulfhydryl modifier pCMBS produced irreversible functional changes. Based on drug-specific inhibition of pCMBS modification, we infer a number of anesthetic contact residues: ETO binds near $\beta 3M286$, $\beta 3F289$, and $\beta 3V290$ (Fig 5A); PRO binds near $\beta 3M286$ and $\beta 3V290$ (Fig 5B); and ALX binds near $\beta 3V290$, $\beta 3F293$, $\beta 3L297$, and $\beta 3F301$ (Fig 5C). Mapping these

residues onto our $\alpha 1\beta 3\gamma 2L$ structural model (Figs 5D–I) suggests that all three anesthetics bind in transmembrane $\beta^+-\alpha^-$ inter-subunit clefts, with overlapping ETO and PRO sites extending from the middle of $\beta 3$ -M3 (near $\beta 3V290$) extracellularly (Figs 5D and E), and the ALX site extending from $\beta 3V290$ intracellularly (Fig 5F).

Alphaxalone and Neurosteroids Bind to Inner Transmembrane $\beta^+-\alpha^-$ Sites

Single-point mutations that affect neurosteroid sensitivity in heteromeric mammalian GABA_A receptors (Table 1) are found throughout the transmembrane $\beta^+-\alpha^-$ cleft. Our SCAMP results for ALX provide evidence of contact with four inner $\beta 3$ -M3 residues facing the $\beta^+-\alpha^-$ interface. The strongest prior evidence for an inner transmembrane $\beta^+-\alpha^-$ neurosteroid site is $\beta 3F301$ photolabeling with 6-AziP²², but the use of homomeric $\beta 3$ receptors and failure to test if neurosteroids block 6-AziP labeling make it far weaker than studies in heteromeric receptors using photolabeling derivatives of ETO and PRO²⁶. Mutations at both $\alpha 1I239$ and $\alpha 1Q242$, located opposite $\beta 3F293$ in our structural model (Fig 1), impair receptor sensitivity to neurosteroids^{13,14,28} and $\alpha 1Q242C$ confers insensitivity to ALX, but not to ETO (our unpublished data). The lack of pCMBS-induced effects in receptors with $\alpha 1I239C$ and $\alpha 1Q242C$ mutations⁶ precludes SCAMP tests, and contrasts with our current findings in inner $\beta 3$ -M3 mutants. Other indirect support for inner transmembrane neurosteroid sites include evidence that a membrane-impermeant steroid positively modulates GABA_A receptors only when applied intracellularly²³. Docking calculations using the $\beta 3$ homomeric GABA_A receptor structure³⁰ also locate pregnanolone and allopregnanolone sites near both $\beta 3F301$ and $\beta 3L297$ ³⁹.

Previous functional, SCAMP, and photolabeling evidence (Table 1) all locate ETO and PRO sites in outer transmembrane $\beta^+-\alpha^-$ clefts. In comparing ALX contacts in $\beta 3$ -M3 with those for ETO and PRO, we found that, with the exception of $\beta 3V290C$, ALX contacts were mutually exclusive with PRO or ETO contacts. We also recently reported that ETO contacts $\alpha 1L232$, and that both ETO and PRO contact $\alpha 1M236$, while ALX contacts neither⁵. Altogether, our current results indicate that ALX binds in inner transmembrane $\beta^+-\alpha^-$ cleft sites abutting outer transmembrane ETO/PRO sites, with possible contact of outer and inner sites near $\beta 3V290$.

Neurosteroids enhance GABA_A receptor photolabeling by ETO derivatives²⁸ and neurosteroid-ETO combinations synergize in both enhancing GABA_A receptor gating and anesthetizing animals²⁹. An allosteric mechanism for this synergy through mutual coupling of sites to channel gating is suggested by our observations that both ETO and PRO accelerate pCMBS modification of $\beta 3F293C$ in the ALX sites, while ALX accelerates pCMBS modification at $\beta 3M286C$ in the ETO/PRO sites. Direct contact between neurosteroids and ETO in abutting sites could also mutually enhance drug binding, contributing to functional synergy.

Propofol and Etomidate Bind to Outer Transmembrane $\beta^+-\alpha^-$ Sites

Our current results extend the map of PRO and ETO contacts on the β^+ aspect of the outer $\beta^+-\alpha^-$ sites (Table 1; Fig 5). Functional and SCAMP results with $\beta 3M286C$ echoed previous studies of $\beta 2M286C$ ^{20,21}. We identified two additional ETO contact residues, $\beta 3F289$ and

β 3V290, while PRO protects β 3V290C but not β 3F289C. Thus, the $\beta^+-\alpha^-$ sites for PRO and ETO overlap, agreeing with previous SCAMP and photolabel competition results (Table 1) ^{5,8,12}. Interestingly, despite evidence that PRO and ETO might contact β 2/3N265 on the M2 helix (Table 1), we found no evidence of contact at β 3T262 or β 3T266 that also abut $\beta^+-\alpha^-$ interfaces in structural models (Fig 1).

Mutant Functional Effects Reflect Allosteric Linkages, Not Drug-Receptor Contacts

The functional effects of both cysteine-substitution and pCMBS modification provide insight into allosteric linkages and aqueous accessibility at the residues we studied. Spanning from M286 to E298, most β 3-M3 cysteine mutations altered GABA EC₅₀ and/or GABA efficacy (Table 2), indicating that this region is coupled to ion channel gating. Similar observations were made in a series of α 1-M1 cysteine-substitutions ⁶. Cysteine mutants throughout β 3-M3 were also accessible to pCMBS, indicating an aqueous pathway extending intracellularly to at least β 3F301, and echoing similar findings on the β 1-M2 helix ⁴⁰.

Mutant functional analyses underlie many of the hypotheses we have tested (Table 1) and it is tempting to infer drug contacts from the altered anesthetic sensitivities of cysteine mutants (Fig 2). However, we recently compared SCAMP with tryptophan mutant drug sensitivity for two photolabeled residues and four anesthetics, finding perfect agreement between SCAMP and photolabeling, but poor concordance with mutant drug sensitivities ⁵. There are multiple other examples of SCAMP identifying anesthetic contacts in GABA_A receptors that weren't photolabeled ^{5,6,26,41}, but only one published report of SCAMP disagreeing with photolabeling ²⁵.

SCAMP Conditionally Reflects Drug-Receptor Contacts

Our SCAMP approach requires functional heterologous receptor expression, quantifiably consistent cysteine modification effects, and drug occupation of a large fraction of sites ²⁶. Even under these conditions, we cannot formally rule out allosteric effects in SCAMP experiments. However, allosteric mechanisms should strongly link the functional effects of different anesthetics to inhibition of modification in relevant mutants. Comparing Figs 2 and 4J, such correlations are absent at many positions where modification was inhibited: F289, V290, F293, and F301. Moreover, drug specificity was demonstrable at every protected cysteine (Fig 4J). Thus, our SCAMP results are more compatible with a steric than an allosteric mechanism for inhibiting pCMBS modification. Inferences of steric interactions between receptor-bound drugs and substituted cysteines are strengthened when protection is concentration-dependent and profound. ALX protection at β 3F293C, β 3L297C, and β 3F301C was relatively weak compared to results for ETO, PRO, and mTFD-MPAB at some of their outer transmembrane contacts ^{5,6,21}. For β 3F293C and β 3L297C, this is attributable to low ALX affinity (see below). The β 3F301C sidechain may be located at the periphery of the steroid site, limiting ALX protection at this position.

In three mutant receptors, α 1 β 3F289C γ 2L, α 1 β 3F293C γ 2L, and α 1 β 3L297C γ 2L, high anesthetic concentrations demonstrated concentration-dependent block of pCMBS modification. In these mutants, weak EC₅₀ enhancement (Fig 2) indicated weak drug binding

based on the Monod-Wyman-Changeux allosteric principle that positive gating modulation reflects the relative affinity of ligands for active (open) vs. inactive (closed) receptors. Thus, weak EC₅₀ enhancement relative to wild-type implies reduced drug affinity for GABA-activated receptors and a need for high drug concentrations to occupy most binding sites. In addition, $\alpha 1\beta 3\gamma 2L$ receptors were characterized by low GABA efficacy, with maximal GABA activating only about 16% of these receptors (Table 2) under control modification conditions. With addition of ETO or PRO, the fraction of activated and desensitized receptors increased, allosterically accelerating $\beta 3\gamma 2L$ modification (Fig 4J). Adding ALX to high GABA likely produced two opposing effects on $\alpha 1\beta 3\gamma 2L$ modification: increased activation/desensitization that accelerates modification, and steric protection that inhibits modification. In initial experiments, 10 μ M ALX produced approximate balance in these opposing effects, while higher ALX concentrations resulted in overall slowing of modification.

Intra-Subunit Pockets

Crystallographic studies of pentameric ligand-gated ion channels reveal that small anesthetics and alcohols can occupy both inter-subunit and intra-subunit transmembrane pockets⁴²⁻⁴⁴. In this study, we examined two mutations, $\beta 3G287C$ and $\beta 3E298C$, that are predicted to face the $\beta 3$ intra-subunit helix bundle pocket, in both outer and inner regions of $\beta 3-M3$ (Fig 1). While we observed altered GABA sensitivity as evidence of pCMBS access and modification in these mutants, no anesthetic protection was observed (Figs 4 and 5) arguing against the presence of positively modulating anesthetic sites in $\beta 3$ intra-subunit pockets.

Conclusions and Significance

Endogenous and synthetic neurosteroids are potent neuromodulators with broad therapeutic potential. Our current SCAMP studies locate positively modulating ALX sites on $\alpha 1\beta 3\gamma 2L$ GABA_A receptors in inner transmembrane $\beta^+-\alpha^-$ inter-subunit clefts. These neurosteroid sites are adjacent to outer transmembrane $\beta^+-\alpha^-$ sites where ETO and PRO act, suggesting both direct and indirect mechanisms for cooperativity between neurosteroids and ETO^{28,29}. Two other outer transmembrane inter-subunit sites, in $\alpha^+-\beta^-$ and $\gamma^+-\beta^-$ clefts, bind PRO and barbiturates^{5,8}. No ligands have yet been identified for the transmembrane $\alpha^+-\gamma^-$ cleft and the inner transmembrane portions of $\alpha^+-\beta^-$ and $\gamma^+-\beta^-$ interfaces, but membrane lipids probably modulate ion channel activity by interacting with transmembrane inter-subunit clefts⁴⁵. In summary, large portions of the five transmembrane inter-subunit clefts in $\alpha 1\beta 3\gamma 2L$ GABA_A receptors are allosterically coupled to ion channel gating. Sub-regions of these clefts form sites for hydrophobic modulators that in several cases, including that of neurosteroids, display remarkable drug selectivity. Structural variations in these inter-subunit interfaces also contribute to subtype-selective GABA_A receptor pharmacology.

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Summary Statement

Substituted cysteine modification-protection indicates that the neurosteroid anesthetic alphaxalone contacts $\alpha 1\beta 3\gamma 2\text{L}$ GABA_A receptors within transmembrane $\beta^+-\alpha^-$ inter-subunit sites that are adjacent and intracellular to previously mapped sites for the potent anesthetics etomidate and propofol.

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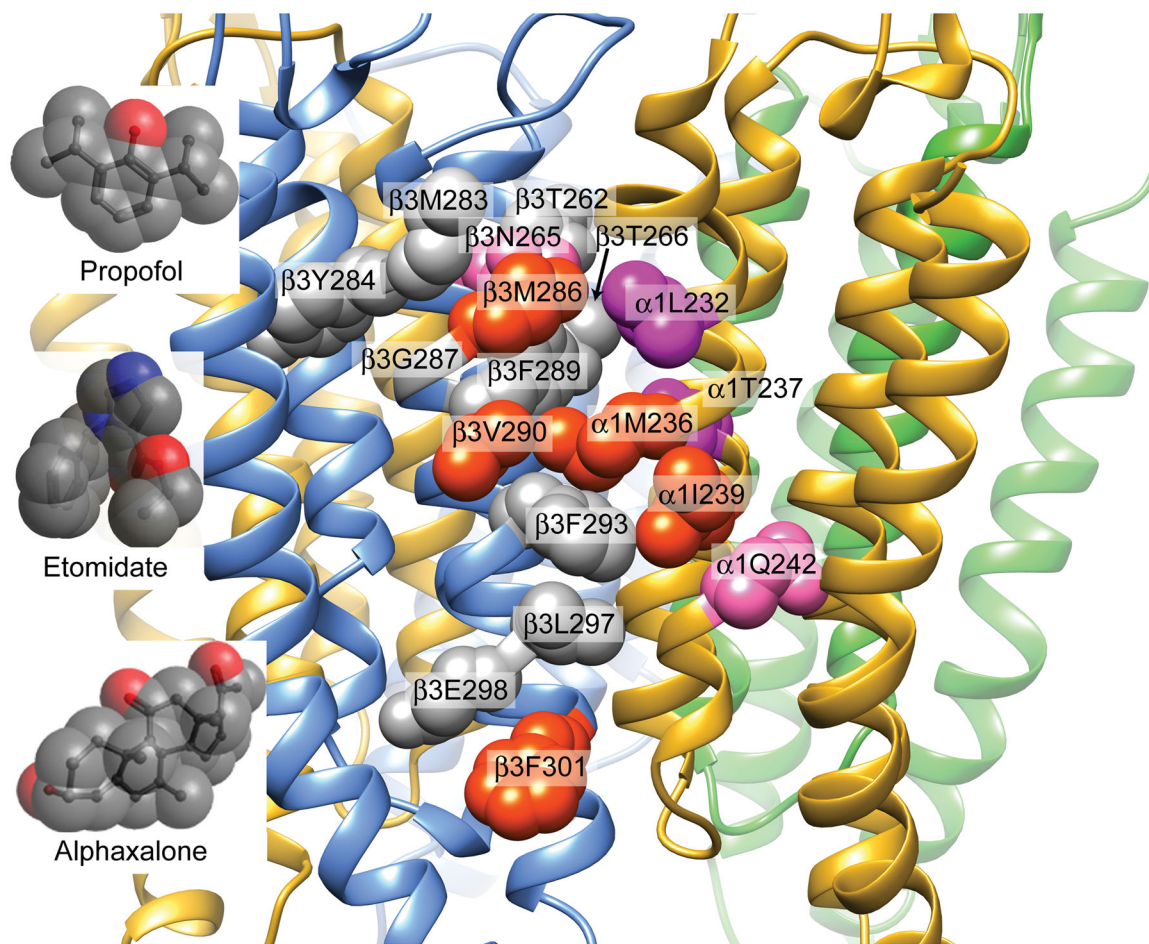


Fig 1. General anesthetic contacts within the GABA_A receptor $\beta^+ - \alpha^-$ transmembrane cleft
 The transmembrane domain of a $\alpha 1\beta 3\gamma 2L$ structural homology model based on GluCl (pdb 4COF) is depicted²⁵. Subunit peptide backbones are shown as ribbons ($\alpha 1$ = yellow; $\beta 3$ = blue; $\gamma 2L$ = green), with sidechains of interest (see Table 1) shown in space-filling mode and labeled. Amino acid sidechains on $\beta 3$ -M3 and $\alpha 1$ -M1 that are directly photolabeled by analogs of one or more study anesthetics are colored orange-red. Anesthetic contact sidechains that have previously been identified using substituted cysteine modification-protection are colored purple. Other $\beta 3$ -M2 and $\beta 3$ -M3 sidechains that line the $\beta^+ - \alpha^-$ cleft and three sidechains predicted to face the $\beta 3$ intra-subunit helix bundle pocket (Y284, G287, and E298), are colored gray. The location of $\alpha 1Q242$ (pink) is also shown. Inserts display the molecular space-filling structures of propofol, etomidate, and alphaxalone, approximately scaled to the receptor model. Hydrogens have been hidden for clarity.

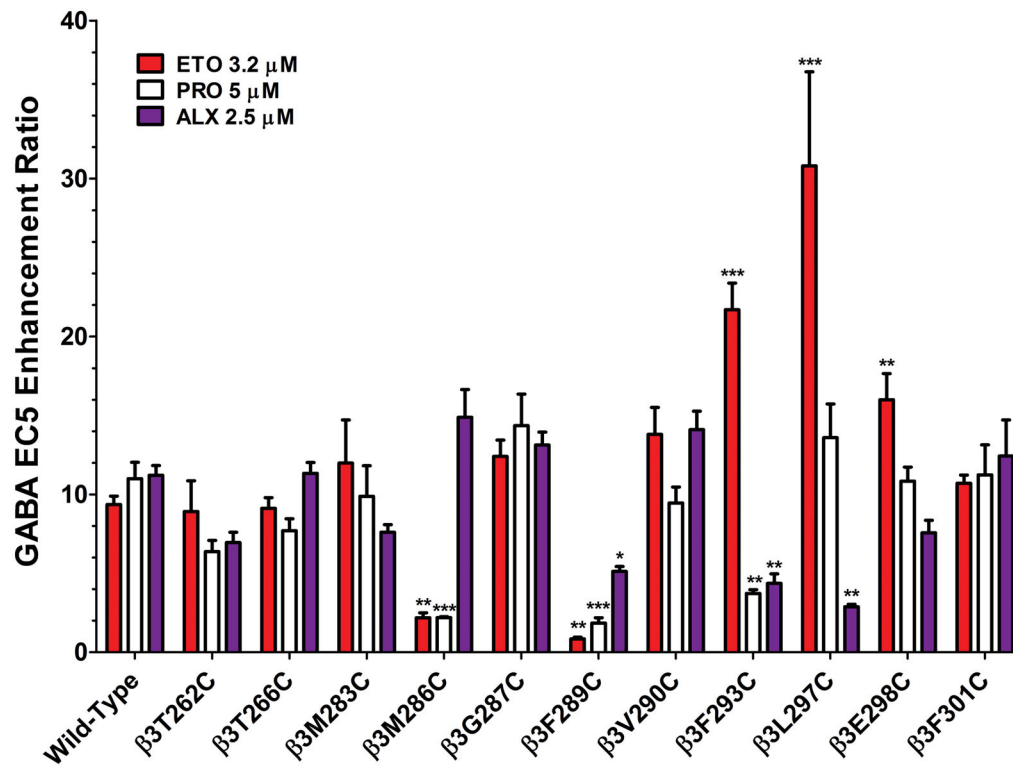


Fig 2. Modulation of wild-type vs. cysteine-substituted GABA_A receptors by etomidate, propofol, and alphaxalone

Each bar represents mean \pm sem results ($n = 4$) of experiments quantifying the anesthetic enhancement of GABA EC5 responses in wild-type and 11 cysteine substituted mutants. The drug concentrations are each 2 x EC50 in tadpole loss of righting reflexes assays and similarly modulate wild-type receptor currents: 3.2 μ M etomidate (ETO, red); 5 μ M propofol (PRO, white); and 2.5 μ M alphaxalone (ALX, purple). Of note, EC5 GABA concentrations were established in comparison with maximal GABA responses. Thus, in mutants where maximal GABA efficacy is low (Table 2), enhancements ratios greater than 20 are possible. Statistically significant differences from wild-type results are indicated by * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

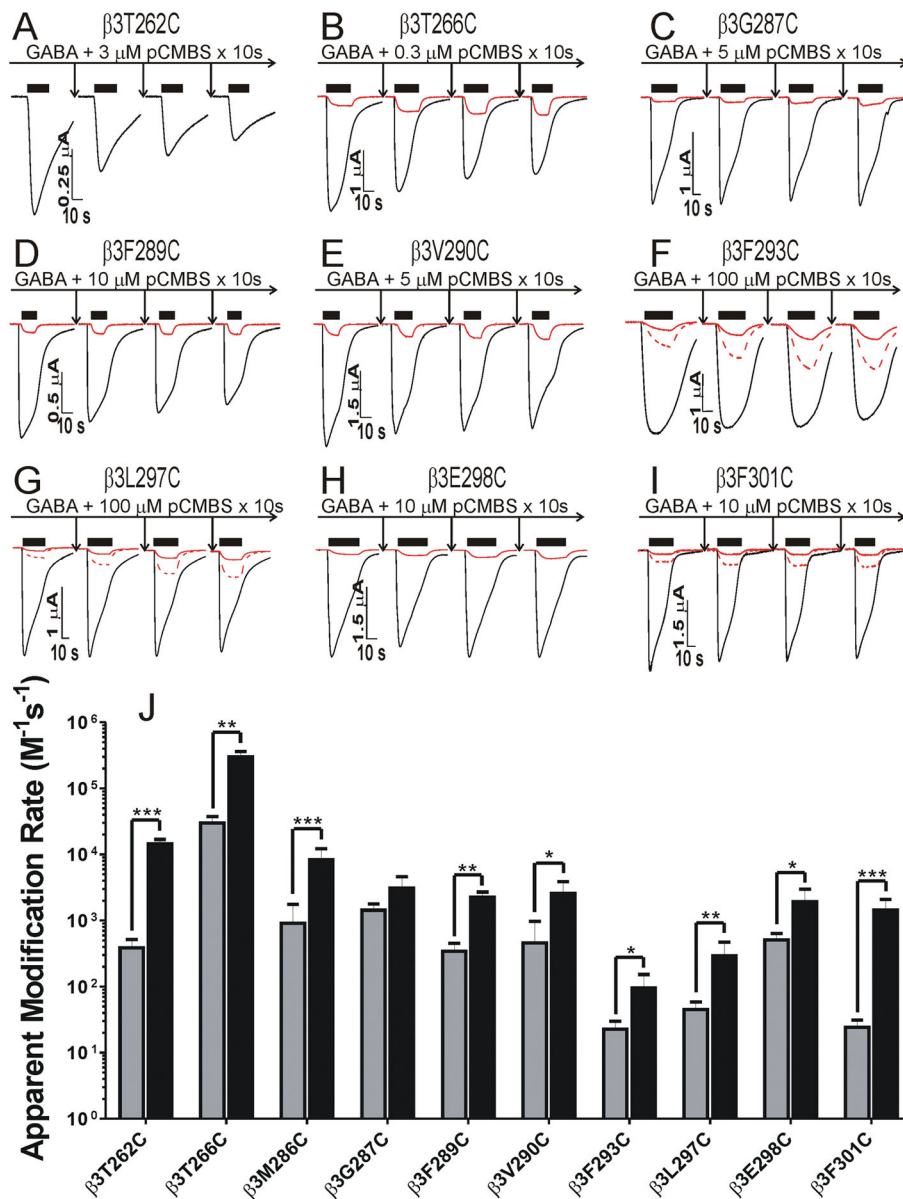


Fig 3. Effects of pCMBS exposure on cysteine-substituted GABA_A receptors in the absence and presence of GABA

Panels A through I, each labeled with the relevant cysteine mutant, show current traces from an oocyte stimulated with either EC5 GABA (red) or maximal GABA (black) before and after three cycles of pCMBS + GABA exposure and ND96 wash. Panel A omits EC5 traces, which diminished in parallel with high GABA responses. EC5 traces in panels F, G, and I are duplicated at 3 x magnitude (red dashed lines) to better illustrate the effects of pCMBS modification. Specific modification conditions are indicated in each panel. GABA exposure periods are indicated by black bars over traces. Panel J summarizes the apparent rates of receptor modification (average \pm sem) in the absence (gray bars) and presence of GABA (black bars). Corresponding examples of rate analyses are shown in Fig 4A through I. With

the exception of β 3G287C, GABA significantly accelerated the apparent modification rates.
* $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$

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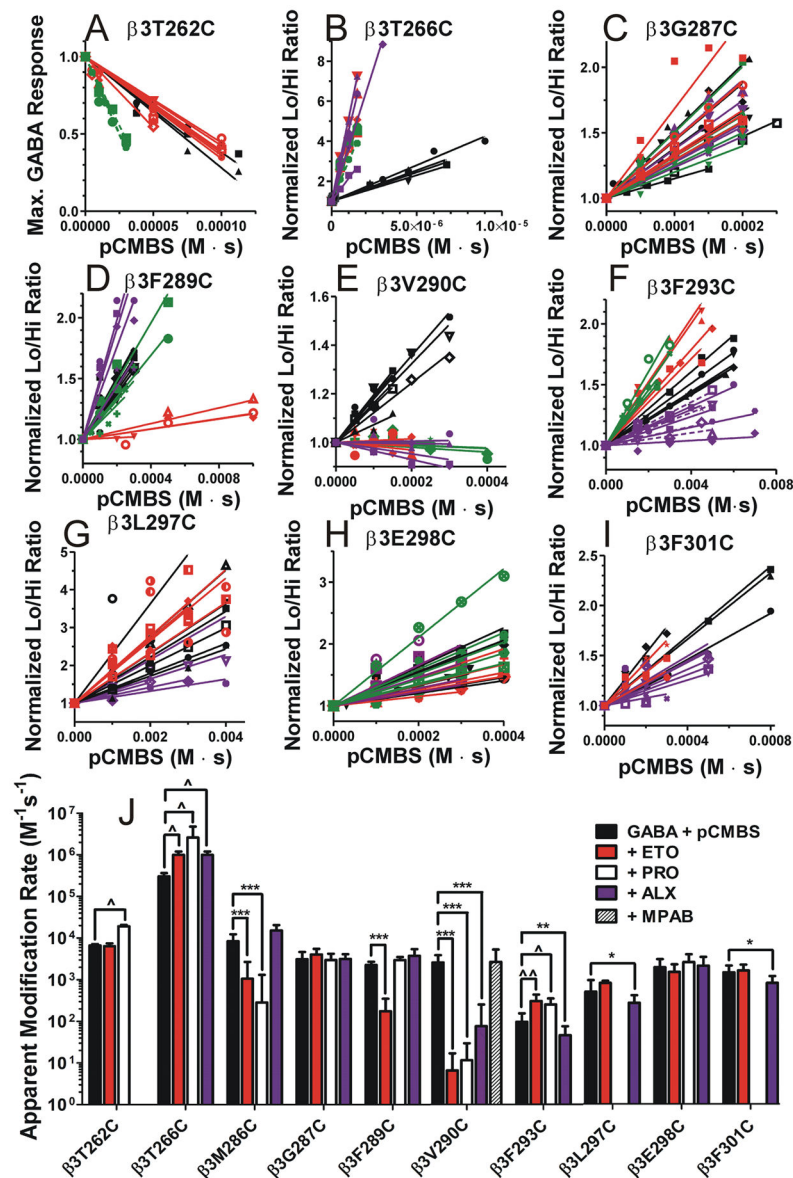


Fig 4. Anesthetic protection of substituted cysteine mutant GABA_A receptors
 Panels A through I, labeled by mutation, show individual oocyte data and linear fits for control modification (GABA + pCMBS; black symbols and lines), and modification in the presence of etomidate (red symbols and lines), propofol (green symbols and lines), and alphaxalone (purple symbols and lines) results. Corresponding example current traces for control modification are shown in Fig. 3. Anesthetic concentrations were 10 μM etomidate, 20 μM propofol, and 10 μM alphaxalone, except for β3F289C, β3F293C, and β3L297C where five-fold higher concentrations were used. Data for β3M286C is not shown, because we have previously reported similar results²¹. Panel J summarizes mean ± SD rates (fitted linear slopes) for all 10 ten cysteine-substituted mutants on a logarithmic scale. Results for 8 μM mTFD-MPAB effects on β3V290C modification (n = 6) are included. Negative slopes for β3T262C and β3V290C were inverted for rate comparisons. Two-way ANOVA analysis was

used to assess whether addition of anesthetics significantly altered the apparent rates of modification relative to control conditions with GABA + pCMBS in each mutant. Protection is inferred in cases where addition of anesthetics significantly reduced modification rates. * indicates significantly reduced modification rate, while ^ indicates significantly increased modification rate: * or ^, $p < 0.05$; ** or ^^, $p < 0.01$; ***, $p < 0.001$.

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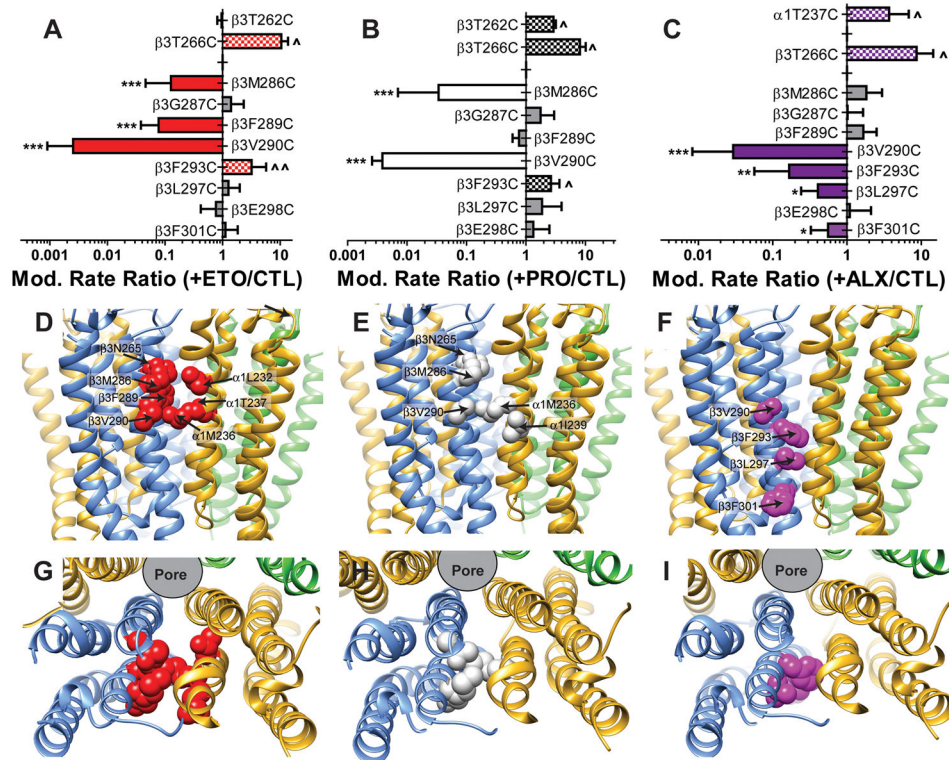


Fig 5. Summary of substituted cysteine modification and protection results by anesthetic drug
 The top row of panels summarizes the ratio of modification rates (mean \pm sd) in the presence vs. absence of anesthetic for each drug at the mutations used in the current study. Cases where no significant change was observed are indicated by gray bars. Cases where anesthetics caused significant slowing of modification are identified by solid colored bars and those where anesthetics produced significant acceleration of modification are identified by checked bars with the same coloring scheme (etomidate = ETO, red; propofol = PRO, white; alphaxalone = ALX, purple). Contact between anesthetics and sidechains is inferred in cases where modification is inhibited. Significance is annotated as described for figure 4J. The middle row of panels depicts the transmembrane domain backbone ribbon structure of our $\alpha 1\beta 3\gamma 2L$ homology model as viewed from the side. Subunit color coding is the same as in Fig 1. Contact residues, based on both photolabeling and substituted cysteine modification-protection studies, are identified for each drug in separate panels as colored and labeled space-filling models. The bottom set of panels depict the same models and contact sidechains viewed from the extracellular space, with the extracellular domains removed.

Table 1Evidence of Neurosteroid and Anesthetic Contacts in β^+ - α^- Transmembrane Interfaces of GABA_A receptors

Residue	Receptor	Mutant Effects ^a	Photolabels ^b	SCAMP ^c
α1L232	α1β3γ2L	ETO, PRO ⁵	—	ETO ^{5,6}
α1M236	α1β3γ2L	ETO, PRO, ALX ^{5,7}	Azi-ETO ⁸	ETO, PRO ^{5,6,9}
	α1β3		Azi-ETO ¹⁰	
	α1β3		TDBzl-ETO ¹¹	
	α1β3		Azi-Pm ¹²	
α1T237	α1β2γ2L	Neurosteroids ¹³		
	α1β3γ2L		—	ETO ⁶
α1I239	α1β2γ2L	Neurosteroids ¹³		
	α1β3γ2L		—	— ^{6, d}
	α1β3		Azi-Pm ¹²	
α1Q242	α1β2γ2L	Neurosteroids ^{13,14}		
	α1β3γ2L		—	— ^{6, d}
β3N265	α1β3γ2L	ETO, PRO ¹⁵⁻¹⁸	—	ETO, PRO ⁹
β3M286	α1β2/3γ2	ETO, PRO ^{7,18,19}	Azi-ETO ^{8,10}	ETO, PRO ^{20,21}
	α1β3		TDBzl-ETO ¹¹	
	α1β3		Azi-Pm ¹²	
β3F289	α1β3γ2L		(ETO) ^{8, b}	
β3V290	α1β3		TDBzl-ETO ¹¹	
β3F301	β3		6-AziP ²²	

ETO = etomidate; PRO = propofol; ALX = alphaxalone; Azi-ETO = azi-etomidate; TD-Bzl-ETO = *p*-trifluoromethyl-diaziryl-phenyl-etomidate; Azi-Pm = *m*-azi-propofol; 6-AziP = 6-azi-pregnanolone; neurosteroids are allopregnanolone (ALLOP) and tetrahydro-deoxycorticosterone (THDOC). — Indicates negative results.

^aDrugs displaying reduced enhancement of submaximal GABA responses in mutant receptors are listed. Not all loci have been tested with ETO, PRO, and ALX. Negative effects of α1M236 and β3M286 mutations on ALX sensitivity have been reported.^{5,18}

^bDirect or indirect (indicated by parentheses) photolabeling evidence is included. Specifically, β3F289 photolabeling by *m*-trifluoromethyl-mephobarbital is inhibited by etomidate.

^cSCAMP is Substituted Cysteine Modification-Protection. Drugs demonstrating protection are listed. Not all loci have been tested with ETO, PRO, and ALX. Negative results have been reported for PRO at α1L232C and ALX at α1L232C, α1M236C and β3M286C.^{5,6,21}

^dApplication of a cysteine modifying reagent (*p*-chloromercuribenzenesulfonate) to α1I239C and α1Q242C did not alter function, precluding protection studies.

Table 2
Functional and Pharmacological Characteristics of α 1 β 3 γ 2L GABA_A Receptors with β 3 Cysteine Substitutions

Receptor Type	GABA EC ₅₀ (μ M) [95% CI] (n)	GABA Efficacy mean \pm se (n)	Spont. Activation mean \pm se (n)	Maximal Effect of pCMBs Modification (range)
α 1 β 3 γ 2L	31 [23 to 41] (8)	0.88 \pm 0.025 (5)	< 0.005 (5)	No effect
α 1 β 3T262C γ 2L	21 [18 to 25] (4)	0.93 \pm 0.03 (4)	< 0.005 (3)	Reduce max current (95–99%)
α 1 β 3T266C γ 2L	143 [130 to 157] (6) ***	0.88 \pm 0.02 (4)	< 0.005 (3)	\uparrow lo/hi GABA response (10 to 13-fold)
α 1 β 3M283C γ 2L	46 [43 to 50] (3)	0.92 \pm 0.023 (3)	< 0.005 (3)	No effect
α 1 β 3M286C γ 2L	148 [122 to 180] (3) ***	0.65 \pm 0.023 (3) **	< 0.005 (3)	\uparrow lo/hi GABA response (5.8 to 7.8-fold)
α 1 β 3G287C γ 2L	78 [67 to 92] (4) **	0.96 \pm 0.034 (4) *	< 0.005 (3)	\uparrow lo/hi GABA response (2.3 to 3.5-fold)
α 1 β 3F289C γ 2L	5.9 [5.3 to 6.7] (4) ***	0.99 \pm 0.02 (4) **	0.034 \pm 0.016 * (4)	\uparrow lo/hi GABA response (2.8 to 4.0-fold)
α 1 β 3Y290C γ 2L	36 [32 to 41] (4)	0.92 \pm 0.03 (4)	< 0.005 (3)	\uparrow lo/hi GABA response (2.3 to 3.1-fold)
α 1 β 3F293C γ 2L	181 [143 to 229] (3) ***	0.16 \pm 0.018 (3) ***	< 0.005 (3)	\uparrow lo/hi GABA response (2.6 to 4.3-fold)
α 1 β 3L297C γ 2L	114 [104 to 125] (4) ***	0.54 \pm 0.07 (5) ***	< 0.005 (4)	\uparrow lo/hi GABA response (5.5 to 7.2-fold)
α 1 β 3E298C γ 2L	103 [88 to 112] (4) ***	0.63 \pm 0.07 (3) ***	< 0.005 (3)	\uparrow lo/hi GABA response (3.5 to 5.5-fold)
α 1 β 3F301C γ 2L	34 [30 to 39] (4)	0.93 \pm 0.04 (4)	< 0.005 (4)	\uparrow lo/hi GABA response (3.8 to 4.9-fold)

Differs from wild-type at:

* p < 0.0045,

** p < 0.0005,

*** p < 0.0001