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Tryptophan and Cysteine Mutations in M1 Helices of $\alpha 1\beta 3\gamma 2L$ γ -Aminobutyric Acid Type A Receptors Indicate Distinct Inter-subunit Sites for Four Intravenous Anesthetics and One Orphan Site

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Abstract

Background—GABA_A receptors mediate important effects of intravenous general anesthetics. Photolabel derivatives of etomidate, propofol, barbiturates, and a neurosteroid incorporate in GABA_A receptor transmembrane helices M1 and M3 adjacent to inter-subunit pockets. However, photolabels have not been consistently targeted at heteromeric $\alpha\beta\gamma$ receptors and don't form adducts with all contact residues. Complementary approaches may further define anesthetic sites in typical GABA_A receptors.

Methods—Two mutation-based strategies, substituted tryptophan sensitivity and substituted cysteine modification-protection, combined with voltage-clamp electrophysiology in *Xenopus* oocytes, were used to evaluate interactions between four intravenous anesthetics and six amino acids in M1 helices of $\alpha 1$, $\beta 3$, and $\gamma 2L$ GABA_A receptor subunits: two photolabeled residues, $\alpha 1M236$ and $\beta 3M227$, and their homologs.

Results—Tryptophan substitutions at $\alpha 1M236$ and positional homologs $\beta 3L231$ and $\gamma 2L246$ all caused spontaneous channel gating and reduced GABA EC₅₀. Substituted cysteine modification experiments indicated etomidate protection at $\alpha 1L232C$ and $\alpha 1M236C$, mTFD-MPAB protection at $\beta 3M227C$ and $\beta 3L231C$, and propofol protection at $\alpha 1M236C$ and $\beta 3M227C$. No alphaxalone protection was evident at the residues we explored and none of the tested anesthetics protected $\gamma 2I242C$ or $\gamma 2L246C$.

Conclusions—All five inter-subunit transmembrane pockets of GABA_A receptors display similar allosteric linkage to ion channel gating. Substituted cysteine modification and protection results were fully concordant with anesthetic photolabeling at $\alpha 1M236$ and $\beta 3M227$, and reveal overlapping non-congruent sites for etomidate and propofol in $\beta^+ - \alpha^-$ interfaces and mTFD-

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MPAB and propofol in $\alpha^+ - \beta^-$ and $\gamma^+ - \beta^-$ interfaces. Our results identify the $\alpha^+ - \gamma^-$ transmembrane interface as a potentially unique “orphan” modulator site.

Keywords

Ligand-gated ion channel; glycine receptor; GluCl; alcohol; general anesthetic; cysteine modification; photolabel; electrophysiology; allosteric modulator; allosteric agonist

Introduction

Etomidate, propofol, alphaxalone, and barbiturates are intravenous general anesthetics that enhance the activity of γ -aminobutyric acid type A (GABA_A) receptors, the dominant inhibitory neurotransmitter receptors in mammalian brain and members of the pentameric ligand-gated ion channel (pLGIC) superfamily^{1–4}. GABA_A receptor subunits contain structural elements common to all pLGICs, including an N-terminal extracellular domain and a transmembrane domain with four alpha helices (M1 to M4). Most GABA_A receptors consist of two α , two β , and one γ subunit arranged $\beta\alpha\beta\alpha\gamma$ counterclockwise⁵, creating four distinct types of subunit interfaces: $\alpha^+ - \beta^-$, $\alpha^+ - \gamma^-$, $\beta^+ - \gamma^-$, and two $\beta^+ - \alpha^-$ (Fig 1).

Anesthetic photolabels form adducts with GABA_A receptor residues that are homologs of ivermectin contacts in transmembrane inter-subunit pockets of GluCl pLGICs, imaged with x-ray crystallography⁶. Azi-etomidate labeled α M236 in α -M1 and β M286 in β -M3 in GABA_A receptors from bovine brain or cells expressing $\alpha 1\beta 3\gamma 2$ L^{7,8}. The barbiturate *R*-5-allyl-1-methyl-5-(*m*-trifluoromethyl-diazirinyphenyl) barbituric acid (mTFD-MPAB), photolabeled residues in β 3-M1 (β 3M227), α 1-M3, and γ 2-M3⁸. *m*-Azi-propofol (azi-Pm) photolabeled both α 1M236 and β 3M227 in $\alpha 1\beta 3$ receptors⁹. 6-Azi-pregnanalone (6-aziP), a neurosteroid derivative, photolabeled β 3F301 in $\beta 3$ homomeric receptors¹⁰. Propofol, but not alphaxalone, displaced both azi-etomidate and mTFD-MPAB labeling in $\alpha 1\beta 3\gamma 2$ L receptors^{8,11,12}. These results suggest that $\alpha 1\beta 3\gamma 2$ L receptors form overlapping etomidate and propofol sites in $\beta 3^+ - \alpha 1^-$ interfaces adjacent to $\alpha 1$ -M1s, and overlapping mTFD-MPAB and propofol sites in homologous $\alpha 1^+ - \beta 3^-$ and $\gamma 2^+ - \beta 3^-$ pockets abutting $\beta 3$ -M1s (Fig 1). However, we don't know how propofol sites overlap with vs. differ from etomidate and mTFD-MPAB sites, because photolabels don't adduct all residues that contact parent drugs^{13,14}. Additionally, no anesthetics have photolabeled $\gamma 2$ -M1, and because receptors photolabeled by aziPm and 6-aziP lacked $\gamma 2$ subunits, propofol or neurosteroid interactions with $\gamma 2$ remain untested.

To both complement and supplement photolabeling data, two approaches based on voltage-clamp electrophysiological studies of GABA_A receptors with mutations at putative anesthetic contact residues have been widely applied. Tryptophan substitutions in putative anesthetic sites have been reported to both mimic the channel-gating effects of anesthetic binding and impair anesthetic modulation^{15–19}. Cysteines substituted at putative contact residues have been covalently modified by probes such as *p*-chloromercuribenzenesulfonate (pCMBS) and protected from modification by anesthetics^{14,20–24}. However, neither of these approaches has been rigorously compared to photolabeling.

In the current study, we created tryptophan and cysteine mutations at two photolabeled residues in M1 helices, $\alpha 1$ M236 and $\beta 3$ M227, and their homologs based on sequence alignment: $\alpha 1$ L232, $\beta 3$ L231, $\gamma 2$ I242 and $\gamma 2$ L246 (Table 1). At each locus, we assessed tryptophan mutant functions and performed substituted cysteine modification-protection (SCAMP) experiments to probe interactions with etomidate, propofol, alphaxalone, and mTFD-MPAB. Our analysis addressed three key questions: 1) Do SCAMP results and/or tryptophan mutant sensitivity results for the study drugs agree with photolabeling at $\alpha 1$ M236 and $\beta 3$ M227? 2) Do the $\alpha 1$ -M1 contacts for etomidate and propofol or the $\beta 3$ -M1 contacts for mTFD-MPAB and propofol differ? 3) Do alphaxalone, propofol, or other anesthetics bind in the $\alpha 1^+ - \gamma 2^-$ interface?

Materials and Methods

Animals

Female *Xenopus* frogs were used as a source of oocytes in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Approval for animal use in this study was obtained from 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 prior to oocyte collection. All efforts were made to minimize animal suffering.

Materials

R-mTFD-MPAB 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 to 4, 8, or 16 μ M for experiments. Alphaxalone was purchased from Tocris Bioscience (Bristol, UK), stored as a 10 mM solution in DMSO, and diluted to 1.25, 2.5, or 5 μ M in electrophysiology buffer for experiments. Propofol (2,6-diisopropylphenol) was purchased from Sigma-Aldrich (St. Louis, MO, USA), stored as a 10 mM solution in DMSO and diluted to 2.5, 5, or 10 μ M 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 to 1.6, 3.2, or 6.4 μ M in electrophysiology buffer for experiments. We have previously shown that DMSO and propylene glycol at the dilutions used here produce no effects on GABA_A receptor function²³. Para-chloromercuribenzenesulfonic acid sodium salt (pCMBS) was purchased from Toronto Chemical Research (Toronto, ON, Canada) and fresh 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 described¹⁵. Complementary DNAs encoding human $\alpha 1$, $\beta 3$, and $\gamma 2$ L GABA_A receptor subunits in pCDNA3.1 expression vectors (Thermo Fisher Scientific, Waltham, MA, USA) were used as mutagenesis templates. Tryptophan and cysteine mutations were introduced into cDNAs by site-directed

mutagenesis using QuikChange kits (Agilent Technologies, Santa Clara, CA, USA). Selected clones were sequenced through the entire coding region, and a single clone for each mutant was chosen for subsequent use. Messenger RNAs were synthesized on linearized DNA templates using mMessage Machine kits (Ambion Thermo Fisher), purified, and mixed in a ratio of 1 α :1 β :3 γ , then diluted in RNAase-free water to 1 ng/nl. Oocytes were injected with ~12 ng total mRNA mix and incubated in ND96 buffer (in mM: 96 NaCl, 2 KCl, 1 CaCl₂, 0.8 MgCl₂, 1 EGTA, 10 HEPES, pH 7.5) supplemented with ciprofloxacin (2 mg/ml) and amikacin (100 μ g/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 room temperature (21 to 23 °C). Oocytes were positioned in a low volume (30 μ l) custom-built flow-cell and impaled with two borosilicate glass micro-electrodes filled with 3 M KCl (resistance < 1 M Ω). Oocytes were voltage-clamped at -50 mV (model OC-725C, Warner Instruments, Hamden CT, USA). Superfusion solutions based on ND96 were selected 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). Solutions were selected by activating electrical valves (VC-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 the delivery of different superfusion solutions and recordings of voltage and current signals. Currents were filtered at 1 kHz, digitized at 100 Hz, and stored on a computer disk for offline analysis.

GABA concentration-responses

Currents in voltage-clamped oocytes expressing GABA_A receptors were exposed to solutions containing GABA (range 0.1 μ M to 10 mM) with or without anesthetics for 10 to 20 s, followed by 5 minute ND96 wash. Normalization sweeps at 1 to 10 mM GABA alone, (i.e. maximum GABA for the specific receptor) were recorded every other or every third experiment. GABA concentration-responses in the presence of etomidate or mTFD-MPAB were studied using the same method with solutions of variable GABA combined with 3.2 μ M etomidate or 8 μ M mTFD-MPAB. Baseline responses were measured using anesthetic without GABA. Oocytes were not pre-exposed to anesthetics when co-applied with GABA, and maximal normalization sweeps were in either high GABA alone or high GABA plus anesthetic. At least 3 oocytes from two different frogs were used for each concentration response.

Spontaneous receptor activity and maximal GABA efficacy

Spontaneous activation of GABA_A receptors (in the absence of GABA or anesthetics) was assessed by applying 2 mM picrotoxin to voltage-clamped oocytes. Outward currents that reversed during picrotoxin washout were assumed to represent spontaneously active channels. Spontaneous activity, if detected, was normalized to maximally activated inward GABA-elicited current in the same cell (n = 3 cells).

Maximal GABA efficacy for each receptor was determined by comparing peak currents elicited with high GABA to currents elicited with high GABA supplemented with 2.5 μ M alphaxalone, which positively modulated all receptors in this study. GABA efficacy was calculated by normalizing GABA responses to GABA + alphaxalone responses, which we assumed represents 100% activation, in the same cell ($n = 3$ cells).

GABA EC5 enhancement

Voltage-clamped oocytes expressing wild-type or mutant 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 peak 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 $2 \times$ EC50 for loss-of-righting-reflexes (LoRR) in tadpoles: 2.5 μ M alphaxalone²⁵, 3.2 μ M etomidate²⁶, 5 μ M propofol²⁷, or 8 μ M mTFD-MPAB²⁸. For each receptor type (wild-type and 12 mutants) and four anesthetics, triplicate measurements of current response to GABA EC5 and GABA EC5 + anesthetic were obtained in at least four oocytes from two different frogs. Because receptors with the γ 2L246W mutation displayed substantial spontaneous activity, $2 \times$ EC50 anesthetic enhancement of GABA EC5 responses approached maximal GABA responses for all drugs. This receptors was also studied using $1 \times$ EC50 for tadpole LoRR of each anesthetic.

Substituted Cysteine Modification and Protection (SCAMP)

Cysteine modification and protection were performed in the presence of maximally activating GABA, as previously described²³. Maximal GABA serves to 1) enhance anesthetic binding site occupancy in protection experiments, 2) assure in most cases that the mix of receptor states is similar in both control modification and protection studies, and 3) increase the rate of sulfhydryl modification. Each substituted cysteine mutant receptor was expressed in *Xenopus* oocytes, voltage clamped, and exposed to both GABA EC5 (low) and a maximally activating GABA concentration (high). After 5 minute wash, oocytes were then exposed for 30 s to ND96 containing high GABA plus pCMBS, a water-soluble cysteine modifying reagent, ranging in concentration from 1 μ M to 1 mM, followed by 5 minute wash in ND96. Responses to low and high GABA were then repeated. We thus confirmed that pCMBS produced irreversible changes in receptor function (in most cases an increase in the low:high GABA response ratio), and identified pCMBS concentrations suitable for comparing initial rates of modification. For control modification rate experiments, voltage-clamped oocytes were repeatedly tested for responses to both EC5 and maximal GABA, then washed for 5 min in ND96, to assure that the response ratio was stable (< 5% change) before pCMBS exposure. Oocytes were then exposed for 5 to 10 s to solutions of maximal GABA plus pCMBS at concentrations estimated to produce about 10% of the maximal modification effect, 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. A final modification cycle was performed using $10 \times$ pCMBS concentration for 20 s to complete modification of receptors, and electrophysiological response was assessed as the maximal modification effect.

For anesthetic protection experiments, a similar experimental protocol was used, except that oocytes were exposed to anesthetic for 30 s just before exposure to a solution of pCMBS + GABA + anesthetic. Post-modification wash and response tests were identical to control conditions (no anesthetic present). Anesthetic concentrations used in protection studies were 2 to 4 × LoRR EC₅₀. For each cysteine mutant, at least 5 oocytes were studied in control modification experiments and at least 4 oocytes were studied in protection experiments. Group sample sizes were based both on prior experience and a power analysis performed with G*Power 3.08 software (Franz Faul, Universitat Kiel, Germany). Based on our experience with SCAMP, SD/mean ratios for modification rates range from 0.2 to 0.5 and in protection experiments we aimed to detect drops of more than 50% from control modification rates. We estimated sample sizes for two groups with respective means of 2 and 1, both with SD = 0.4 (an effect size of 2.5), in a one-tail t-test with $\alpha = 0.013$ (adjusted for four drug comparisons to control) and $\beta = 0.85$, indicating a need for 5 samples per group.

Data analysis and statistics

Results in text and figures are mean ± 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):

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

where EC₅₀ is the half-maximal activating GABA concentration, and nH is the Hill slope. Mean GABA EC₅₀ and 95% confidence interval are reported. GABA EC₅₀ shifts in the presence of anesthetics were calculated from the difference in log(EC₅₀) values (control – anesthetic) with propagation of log(SD) errors²⁹. Mean GABA EC₅₀ shift ratios and 95% confidence intervals were calculated. Statistical comparison of GABA EC₅₀s and anesthetic shifts between mutants and wild-type was based on the calculated confidence intervals of the differences of means.

Functional characteristics of mutant receptors—Comparison of both spontaneous activity and GABA efficacy between mutants and wild-type was based on one-way ANOVA with post-hoc Dunnett's tests (in Prism 5.02). EC₅ enhancement data for the four equipotent anesthetic concentrations in wild-type and all six tryptophan mutants was tabulated and analyzed with two-way ANOVA and Bonferroni posttests for wild-type vs. mutation for each anesthetic (Prism 5.02).

SCAMP—Apparent pCMBS modification rates were calculated from data for individual oocytes expressing cysteine mutants. Either normalized maximal GABA response (for $\alpha 1\beta 3\text{M}227\text{C}\gamma 2\text{L}$) or normalized low:high GABA response ratio (for the other five cysteine mutants) was plotted against cumulative pCMBS exposure (M×s) and linear least squares

analyses with y-axis intercepts fixed at 1.0 were applied. For each mutant, the resulting slopes from control and anesthetic protection studies were tabulated and compared using one-way ANOVA in Prism 5.02.

Comparison of photolabeling with mutation-based approaches at $\alpha 1M236$ and $\beta 3M227$ —We treated photolabeling results as “gold standard” evidence of anesthetic contact with a residue. For each anesthetic at each residue studied, we categorized substituted tryptophan sensitivity as positive if EC5 enhancement was significantly reduced compared to wild-type. SCAMP results were categorized as positive if addition of anesthetic significantly reduced the initial rate of modification by pCMBS. We constructed 2×2 contingency tables and calculated the percentage agreement between each mutation-based method and photolabeling (i.e. concordance of positive and negative results), as well as Cohen’s kappa, a measure of agreement between methods that corrects for chance. Contingency analysis with Fisher’s exact test was used to calculate a conservative p-value, with the implicit null hypothesis being that mutation-based results are uncorrelated with photolabeling.

Statistical significance was inferred for $p < 0.05$.

Results

Modulation of wild-type $\alpha 1\beta 3\gamma 2L$ receptors by four intravenous general anesthetics

We expressed GABA_A receptors in *Xenopus* oocytes and characterized their function using two-electrode voltage clamp, assessing GABA concentration-response, maximal GABA efficacy, spontaneous activation, and modulation by intravenous general anesthetics (Table 2). To quantitatively compare the effects of multiple general anesthetics in GABA_A receptors, we used equipotent drug concentrations based on loss-of-righting-reflexes (LoRR) tests in tadpoles. The EC50s for LoRR are 1.6 μ M etomidate, 2.5 μ M propofol, 4 μ M mTFD-MPAB, and 1.25 μ M alphaxalone^{25–28}. Wild-type $\alpha 1\beta 3\gamma 2L$ GABA responses were similarly enhanced by equipotent $2 \times$ EC50 anesthetic concentrations, measured by either the shift in GABA EC50s (Fig 2 shows results for etomidate and mTFD-MPAB) or enhancement of receptor activation at EC5 GABA (Fig 3 shows results for all four anesthetics).

Functional characteristics and anesthetic sensitivity of $\alpha 1$ -M1 Trp substitutions: $\alpha 1M236W\beta 3\gamma 2L$ and $\alpha 1L232W\beta 3\gamma 2L$ receptors

Photolabel analogs of both etomidate and propofol form adducts with $\alpha 1M236$ ^{7,9}. We have previously described some of the functional characteristics of $\alpha 1M236W\beta 2\gamma 2L$ GABA_A receptors¹⁵, which mimic the effects of bound anesthetic, including increased sensitivity to GABA, spontaneous channel activity, as well as reduced modulation by etomidate, quantified as the ratio of GABA EC50s in the absence vs. presence of anesthetic. We hypothesized that tryptophan substitutions within anesthetic binding sites may, as a general rule, mimic the effects of anesthetics and reduce modulation by drugs that occupy those sites.

The functional characteristics of $\alpha 1M236W\beta 3\gamma 2L$ receptors were very similar to those of $\alpha 1M236W\beta 2\gamma 2L$ (Table 2), including very weak modulation by etomidate (Figs 2 and 3). mTFD-MPAB potently activated $\alpha 1M236W\beta 3\gamma 2L$ receptors, while inducing a smaller GABA EC₅₀ shift than in wild-type receptors (Fig 2, Table 2). This may be due to this receptor's spontaneous activity. However, EC5 enhancement by mTFD-MPAB was similar in wild-type and $\alpha 1M236W\beta 3\gamma 2L$ receptors (Fig 3). Propofol, and unexpectedly alphaxalone, also produced significantly less EC5 enhancement in this mutant than in wild-type (Fig 3).

The $\alpha 1$ homolog of $\beta 3M227$ is $\alpha 1L232$ (Table 1). A tryptophan mutation at this site was described in an earlier study of volatile anesthetic modulation³⁰. Oocyte-expressed $\alpha 1L232W\beta 3\gamma 2L$ receptors were characterized by a low GABA EC₅₀, low GABA efficacy, and no spontaneous activation (Table 2). Based on GABA EC₅₀ shifts or EC5 enhancement metrics, modulation of $\alpha 1L232W\beta 3\gamma 2L$ receptors by etomidate and propofol was significantly reduced, while modulation by mTFD-MPAB and alphaxalone was similar to wild-type (Table 2, Figs 2 and 3).

Functional characteristics and anesthetic sensitivity of $\beta 3$ -M1 Trp substitutions: $\alpha 1\beta 3M227W\gamma 2L$ and $\alpha 1\beta 3L231W\gamma 2L$ receptors

Both mTFD-MPAB and aziPm form photo-adducts with $\beta 3M227$ ^{8,9}. The effects of mutations at $\beta 3M227$ and $\beta 3L231$ have not been reported previously. Oocyte-expressed $\alpha 1\beta 3M227W\gamma 2L$ receptors displayed no detectable spontaneous activity, GABA EC₅₀ about twice that of wild-type, and high GABA efficacy (Table 2). Anesthetic modulation of $\alpha 1\beta 3M227W\gamma 2L$ receptors was similar to that in wild-type for etomidate, propofol, and alphaxalone, but significantly reduced for mTFD-MPAB (Table 2, Figs 2 and 3).

The $\beta 3$ homolog of $\alpha 1M236$ is $\beta 3L231$ (Table 1). The functional characteristics of $\alpha 1\beta 3L231W\gamma 2L$ receptors included spontaneous channel gating similar to that in $\alpha 1M236W\beta 3\gamma 2L$, a low GABA EC₅₀, and high GABA efficacy (Table 2). Unexpectedly, etomidate modulation of $\alpha 1\beta 3L231W\gamma 2L$ receptors was significantly less than in wild-type, while modulation by mTFD-MPAB, propofol and alphaxalone was comparable to effects in wild-type (Table 2, Figs 2 and 3).

SCAMP in $\alpha 1$ -M1: $\alpha 1M236C\beta 3\gamma 2L$ and $\alpha 1L232C\beta 3\gamma 2L$ receptors

Basal leak and both low and high GABA responses in voltage clamped oocytes expressing wild-type $\alpha 1\beta 3\gamma 2L$ GABA_A receptors were unaffected by exposure to 1 mM pCMBS for up to 60 s (n = 4 oocytes; data not shown). We have previously described the functional effects of $\alpha 1M236C$ mutations in GABA_A receptors (see Table 3), the effects of pCMBS modification at $\alpha 1M236C$, and evidence that both etomidate and propofol protect this substituted sidechain from modification, while alphaxalone does not^{21,31}. For our current experiments, we first tested $\alpha 1M236C\beta 3\gamma 2L$ receptors for sensitivity to anesthetics, confirming that GABA EC5 responses are enhanced similarly by all four study drugs (Table 3). For modification and protection experiments, we assessed low:high GABA response ratios, which increased by 8.9 ± 0.38 -fold (n = 6) after maximal pCMBS modification. Compared to previous studies, we used lower concentrations of etomidate and propofol in

protection experiments, and also tested protection with 8 μM mTFD-MPAB. In agreement with our prior studies, we found that addition of 2.5 μM alphaxalone dramatically increased the pCMBS modification rate relative to that in the presence of GABA alone (Fig 4). This is explained by observations that GABA alone activates only about 24% of $\alpha 1\text{M}236\text{C}\beta 3\gamma 2\text{L}$ receptors, while pCMBS modification of this receptor is much faster in GABA-activated versus inactive receptors²¹. Therefore, we used pCMBS + GABA + alphaxalone as the control condition for protection experiments with pCMBS + GABA + other anesthetics. These indicated that both etomidate and propofol protect $\alpha 1\text{M}236\text{C}$, while mTFD-MPAB does not (Fig 4).

We and others have also previously described the functional effects of $\alpha 1\text{L}232\text{C}$ mutations (see Table 3) and evidence that etomidate protects this substituted sidechain from pCMBS modification^{21,32,33}. All four anesthetics similarly enhanced GABA EC5 responses in $\alpha 1\text{L}232\text{C}\beta 3\gamma 2\text{L}$ receptors (Table 3). By systematically testing various pCMBS concentrations, we found that very low concentrations (1 μM) of pCMBS produced irreversible enhancement of gating in $\alpha 1\text{L}232\text{C}\beta 3\gamma 2\text{L}$ receptors (Fig 5A and B), in contrast to the inhibitory effects of 200 to 500 μM pCMBS that were previously reported^{21,33}. Maximal pCMBS modification increased the low:high GABA response ratio by 7.1 ± 0.47 -fold ($n = 7$). In comparison to pCMBS + GABA, addition of etomidate significantly slowed modification of $\alpha 1\text{L}232\text{C}\beta 3\gamma 2\text{L}$ receptors (Fig 5B and C). Alphaxalone, propofol, and mTFD-MPAB did not significantly affect the rate of pCMBS modification in this mutant.

SCAMP in $\beta 3\text{-M}1$: $\alpha 1\beta 3\text{M}227\text{C}\gamma 2\text{L}$ and $\alpha 1\beta 3\text{L}231\text{C}\gamma 2\text{L}$ receptors

Studies of cysteine substitutions at $\beta 3\text{M}227$ and $\beta 3\text{L}231$ have not been reported previously. The functional properties of $\alpha 1\beta 3\text{M}227\text{C}\gamma 2\text{L}$ receptors are summarized in Table 3. Modulation of EC5 responses in $\alpha 1\beta 3\text{M}227\text{C}\gamma 2\text{L}$ receptors by etomidate and other study drugs was similar to that in wild-type. The function of oocyte-expressed $\alpha 1\beta 3\text{M}227\text{C}\gamma 2\text{L}$ receptors was irreversibly modified by pCMBS (100 μM ; Fig 6A and B), but unlike other cysteine mutants in this study, modification reduced receptor activation without altering GABA sensitivity (the ratio of responses to EC5 vs. high GABA remained constant). Maximal pCMBS modification reduced peak currents by $71 \pm 5.4\%$ ($n = 6$). Protection experiments showed that both mTFD-MPAB and propofol significantly slowed modification of $\alpha 1\beta 3\text{M}227\text{C}\gamma 2\text{L}$ receptors, while alphaxalone and etomidate did not (Fig 6C).

The functional properties of $\alpha 1\beta 3\text{L}231\text{C}\gamma 2\text{L}$ receptors are summarized in Table 3. This mutant was modulated similarly by all four anesthetics at equipotent concentrations. Modification by pCMBS resulted in enhanced GABA sensitivity based on the ratio of electrophysiological currents elicited with low vs. high GABA (Fig 6D and E). Maximal pCMBS modification increased this ratio 5.7 ± 0.44 -fold ($n = 6$). Anesthetic protection experiments showed strong protection by mTFD-MPAB, but not by etomidate, propofol, or alphaxalone (Fig 6F).

Comparison of tryptophan anesthetic sensitivity and SCAMP results with photolabeling at $\alpha 1M236$ and $\beta 3M227$

Concordance between photolabeling, substituted tryptophan anesthetic sensitivity and SCAMP was assessed for the four anesthetics at the two photolabeled residues that we studied: $\alpha 1M236$ and $\beta 3M227$. In this set of eight anesthetic-residue pairs, there were four positive photolabeling pairs and four negative pairs. Substituted tryptophan sensitivity was categorized as positive if EC50 enhancement by anesthetic in the mutant was significantly lower than in wild-type (Fig 3). SCAMP was categorized as positive if the anesthetic significantly slowed the initial rate of pCMBS modification at the substituted cysteine (Fig 4 and Fig 6C). The categorized results for all three methods are summarized in Table 4. The agreement between photolabeling and substituted tryptophan sensitivity was 75% (Cohen's kappa = 0.5; $p = 0.49$ by Fisher's exact test), with mismatches for propofol- $\beta 3M227$ (a false negative) and alphaxalone- $\alpha 1M236$ (a false positive) interactions. The concordance between photolabeling and SCAMP results was 100%, and statistically significant (Cohen's kappa = 1.0; $p = 0.029$ by Fisher's exact test).

Functional characteristics and anesthetic sensitivity of $\gamma 2-M1$ Trp substitutions: $\alpha 1\beta 3\gamma 2I242W$ and $\alpha 1\beta 3\gamma 2L246W$ receptors

Both substituted tryptophan sensitivity and SCAMP were used to determine if any of the study anesthetics bind near the $\gamma 2-M1$ helix.

Effects of mutations at $\gamma 2I242$ and $\gamma 2L246$ have not been reported previously. The functional properties of $\alpha 1\beta 3\gamma 2I242W$ and $\alpha 1\beta 3\gamma 2L246W$ receptors are summarized in Table 2. The $\gamma 2I242W$ mutation did not produce spontaneous receptor activation or enhance GABA sensitivity. Instead we observed a high GABA EC₅₀ and reduced GABA efficacy in this mutant (Fig 7A). Oocyte-expressed $\alpha 1\beta 3\gamma 2L246W$ receptors were characterized by ~11% spontaneous channel activation, low GABA EC₅₀, and very high GABA efficacy (Table 2; Fig 7B). Modulation of $\alpha 1\beta 3\gamma 2I242W$ by all four intravenous general anesthetics was similar to that observed in wild-type receptors (Fig 7C). In $\alpha 1\beta 3\gamma 2L246W$ receptors, all the anesthetics produced a large amount of direct channel activation (e.g. Fig 7B) and GABA EC50 enhancement with pre-exposure to the anesthetic concentrations used in other mutants produced near-maximal activation, reducing our ability to discern differences between drugs. We therefore studied EC50 enhancement with half the usual anesthetic concentrations (i.e. $1 \times EC_{50}$ for tadpole LoRR; Fig 7C). Under these altered conditions, the overall amount of enhancement was lower, and results indicated that none of the four anesthetics produced significantly more or less enhancement than the others.

SCAMP in $\gamma 2-M1$: $\alpha 1\beta 3\gamma 2I242C$ and $\alpha 1\beta 3\gamma 2L246C$ receptors

The functional properties of oocyte-expressed $\alpha 1\beta 3\gamma 2I242C$ and $\alpha 1\beta 3\gamma 2L246C$ receptors (Table 3) were similar to wild-type (Table 2). Neither of the $\gamma 2-M1$ cysteine mutations altered sensitivity to modulation by any of the four study anesthetics. Application of 10 μM pCMBS to $\alpha 1\beta 3\gamma 2I242C$ receptors irreversibly enhanced GABA sensitivity (Fig. 8A and B). Maximal pCMBS modification increased the low:high GABA response ratio by 5.8 ± 0.37 -fold ($n = 8$). None of the four anesthetic drugs significantly reduced the rate of pCMBS modification, even at twice the concentration used for most other cysteine mutants

in this study (Fig 8C). Modification of $\alpha 1\beta 3\gamma 2L246C$ receptors required high pCMBS concentrations (500 μM), and also enhanced GABA sensitivity (Fig 8D and E). Maximal pCMBS modification increased the low:high GABA response ratio by 5.0 ± 0.38 -fold ($n = 5$). None of the study anesthetics significantly reduced the rate of pCMBS modification in $\alpha 1\beta 3\gamma 2L246C$ receptors.

Given the negative SCAMP results at both $\gamma 2I242$ and $\gamma 2L246$ with the four intravenous anesthetics, we also tested whether a flexible linear alcohol might bind in the $\alpha^+ - \gamma^-$ interface. Additional SCAMP experiments showed no protection by 120 μM n-octanol in either $\alpha 1\beta 3\gamma 2I242C$ or $\alpha 1\beta 3\gamma 2L246C$ receptors ($n = 4$ each, data not shown).

Discussion

Anesthetic photolabeling data in $\alpha 1\beta 3\gamma 2L$ GABA_A receptors remains limited. We aimed to extend the map of drug contacts and test the functional roles of residues using two mutant-based approaches: substituted tryptophan sensitivity and SCAMP. For two residues, $\alpha 1M236$ and $\beta 3M227$, and four intravenous anesthetics, published photolabeling results were 100% concordant with SCAMP, but only 75% concordant with substituted tryptophan sensitivity. SCAMP experiments also indicated etomidate contact at $\alpha 1L232$ and mTFD-MPAB contact at $\beta 3L231$, but no propofol or alphaxalone contact at these loci. At $\gamma 2I242$ and $\gamma 2L246$, both approaches consistently indicated no anesthetic interactions.

SCAMP fully agrees with photolabeling evidence of selective anesthetic binding to inter-subunit GABA_A receptor sites

SCAMP is based on formation of a covalent bond between a sulfhydryl engineered into the target receptor and a chemical probe. SCAMP is thus analogous to photolabeling in requiring proximity between probe and site. Both photolabeling and functional studies indicate that anesthetics bind preferentially to GABA-bound open and desensitized states^{14,34}. Thus, our experiments were designed to promote formation of GABA-bound receptors with high anesthetic affinity, and were powered to detect large protection effects indicating site occupation. We extended our previous SCAMP studies in $\alpha 1M236C$ and $\alpha 1L232C$ ²¹ to include additional drugs, and investigated four new cysteine mutants in $\beta 3M1$ and $\gamma 2M1$. All six substituted cysteines were accessible to pCMBS, forming covalent adducts that irreversibly altered receptor function. In all but one case, cysteine modification enhanced receptor sensitivity to GABA (increased low:high GABA response ratio; Table 3), echoing the effects of tryptophan substitution at most of these positions (Table 2). The exception was $\beta 3M227C$, where pCMBS modification reduced GABA responses. Notably, the $\beta 3M227W$ mutation reduced GABA sensitivity (Table 2).

SCAMP results for $\alpha 1M236C$ and $\beta 3M227C$ were in perfect accord with both direct and indirect (drug competition) anesthetic photolabeling studies (Table 4). Contingency analysis with Fisher's exact test indicated that SCAMP is a sensitive and specific predictor of photolabeling results for these two residues and four anesthetics (four true positives, four true negatives, no false positives or negatives; $p = 0.029$). We can extend this analysis to include $\beta 3M286$, another residue photolabeled by both azetomidate and aziPm and where SCAMP evidence confirms propofol and etomidate contact, but no interaction with

alphaxalone^{7,9,12,20,22}. Adding these two true positives and one true negative to our Fisher's exact test analysis results in $p = 0.0022$. These results support SCAMP as a reliable technique to test putative anesthetic contact points beyond those identified with photolabels.

SCAMP also has limitations as an approach to mapping ligand-receptor contact residues¹⁴. Cysteine modification is frequently dependent on the receptor state. Establishing conditions where the mix of functional receptor states is similar in both control modification and protection experiments can be challenging²¹. Protection studies may not be feasible or interpretable when cysteine-substitutions induce insensitivity to the ligand (possibly impaired binding) or when exposure to reactive probes causes little or no functional change^{21,31}. To infer steric interference from SCAMP results, allosteric inhibition of cysteine modification must be ruled out. We addressed this issue by studying multiple drugs that produce similar functional effects.

SCAMP identifies additional anesthetic contact residues in $\alpha 1$ -M1 and $\beta 3$ -M1 helices

While $\alpha 1$ L232 has not been photolabeled by anesthetic derivatives, $\beta 3$ L231 was photolabeled by a convulsant barbiturate³⁵. Our current SCAMP results indicate that $\alpha 1$ L232C is protected by etomidate, confirming an earlier study using higher drug concentrations²¹. Neither alphaxalone nor mTFD-MPAB protected $\alpha 1$ L232C from modification. Despite evidence that propofol binds adjacent to $\alpha 1$ -M1 and displaces azetomidate, $\alpha 1$ L232C was not protected by propofol. Thus, etomidate and propofol sites in the $\beta^+ - \alpha^-$ interfaces overlap, but not at $\alpha 1$ L232. Of note, aziPm also photolabels $\alpha 1$ I239⁹, but pCMBS does not affect the function of $\alpha 1$ I239C $\beta 2$ $\gamma 2$ L receptors²¹, making SCAMP studies unfeasible.

Our data indicate that $\beta 3$ L231C is protected by mTFD-MPAB, extending the list of this drug's likely contacts. However, while propofol displaces mTFD-MPAB photolabeling it does not protect $\beta 3$ L231C, again suggesting overlapping but non-congruent sites adjacent to $\beta 3$ -M1. Consistent with photolabeling results, neither etomidate nor alphaxalone protected $\beta 3$ L231C. Considered together, the above SCAMP results indicate that etomidate binds exclusively in the two $\beta^+ - \alpha^-$ interfaces near $\alpha 1$ L232 and $\alpha 1$ M236, while mTFD-MPAB binds exclusively in homologous $\alpha^+ - \beta^-$ and $\gamma^+ - \beta^-$ sites abutting β M227 and β L231. Propofol binds to all four of these sites, adjacent to either $\alpha 1$ M236 or β M227.

Do M1 helix tryptophan substitutions mimic anesthetic binding in GABA_A receptors?

This study extended our earlier analysis of $\alpha 1$ M236W and assessed five more tryptophan mutants in M1 helices. Both $\alpha 1$ M236W and $\beta 2$ M286W mutations mimic the effects of anesthetic binding on GABA_A receptors, including decreased GABA EC₅₀, increased GABA efficacy, and spontaneous channel activation, all of which indicate enhanced channel gating¹⁵. We hypothesized that these effects were produced when tryptophan sidechains partially filled the $\beta^+ - \alpha^-$ interfacial pockets where etomidate binds, and this approach has been applied to other transmembrane residues in putative anesthetic sites¹⁷⁻¹⁹. Both $\beta 3$ L231W and $\gamma 2$ L246W mutations produced effects similar to $\alpha 1$ M236W (Table 2). Thus, tryptophan substitution at the 10th residue after the conserved GYF sequence in any of the M1 helices of $\alpha 1\beta 3\gamma 2$ L receptors (Table 1) produced gating effects that mimic anesthetic

binding. Receptors with $\alpha 1L232W$ mutations also displayed increased apparent GABA sensitivity, but not spontaneous channel activation or GABA efficacy higher than wild-type. On the other hand, $\beta 3M227W$ and $\gamma 2I242W$ produced decreases in GABA sensitivity and none of the W mutations at the 6th residue after the conserved GYF caused spontaneous receptor gating. Overall, these results indicate that the middle segments of all M1 helices of $\alpha 1\beta 3\gamma 2$ receptors are similarly linked to ion channel gating and suggest that all five adjacent inter-subunit pockets, including $\alpha^+ - \gamma^-$, are potential allosteric modulator-agonist sites.

Substituted tryptophan sensitivity does not consistently agree with photolabeling or SCAMP evidence of anesthetic binding interactions

If Trp substitutions in M1 helices occlude adjacent inter-subunit anesthetic sites, they should reduce modulation by anesthetics occupying these pockets. In agreement with photolabeling, $\alpha 1M236W$ reduced receptor sensitivity to both etomidate and propofol, while $\beta 3M227W$ reduced sensitivity to mTFD-MPAB. However, anesthetic sensitivities of $\alpha 1M236W\beta 3\gamma 2L$ and $\alpha 1\beta 3M227W\gamma 2L$ receptors were not in full accord with photolabeling and SCAMP results at these sites and others (Table 4).

Unexpectedly, $\alpha 1M236W$ reduced sensitivity to alphaxalone (Fig 3). Interestingly, Henin et al ³⁶ predicted cholesterol binding sites near all six of the M1 residues we studied, and Hosie et al ³⁷ implicated $\alpha T237$, immediately adjacent to $\alpha M236$, as a contact for neurosteroids, based on altered tetrahydrodeoxycorticosterone sensitivity in mutants. However, SCAMP indicated no alphaxalone protection at $\alpha M236$, and alphaxalone enhances azietomidate photolabeling at $\alpha M236$, implying allosteric effects ¹². Indeed, SCAMP provided no evidence of alphaxalone binding near any of the six residues we studied. Correlation with photolabeling is limited, because photolabeling with 6-aziP used $\beta 3$ homomeric receptors (thus, we lack data for α or γ subunits), and mass spectroscopic analysis missed a sequence including $\beta 3M227$ and $\beta 3L231$ ¹⁰. The combined negative SCAMP and photolabeling results together suggest that mutant effects at $\alpha M236$ and $\alpha T237$ are indirectly (allosterically) coupled to neurosteroid sites.

AziPm photolabeled $\beta 3M227$ ⁹ and SCAMP indicates propofol protection at $\beta 3M227C$ (Fig 6), but $\beta 3M227W$ did not reduce sensitivity to propofol (Figs 2 and 3). To explain this result, we suggest that propofol exerts most of its gating effects through the two sites that it shares with etomidate, and relatively weak effects through sites adjacent to $\beta M227$ that it shares with mTFD-MPAB. Thus, the $\beta 3M227W$ mutation could occlude propofol binding to $\alpha^+ - \beta^-$ and $\gamma^+ - \beta^-$ sites while preserving propofol modulation mediated by $\beta^+ - \alpha^-$ sites. This hypothesis is supported by evidence that $\beta N265$ mutations reduce propofol binding and reduce most of its modulatory effects ^{31,38,39}.

For anesthetic interactions at $\alpha 1L232$ and $\beta 3L231$, SCAMP and substituted tryptophan sensitivity agree in five of eight drug-residue pairs (Table 4). Discordant results were obtained at $\alpha 1L232$ for propofol and at $\beta 3L231$ for both etomidate and mTFD-MPAB.

No anesthetics occupy the α^+ – γ^- transmembrane interface

The extracellular α^+ – γ^- interface of GABA_A receptors forms the high-affinity benzodiazepine site^{40,41}, but no anesthetic photolabels incorporate in γ 2-M1 in the transmembrane portion of this interface. In our current study, neither γ 2I242W nor γ 2L246W mutations selectively altered EC50 enhancement by the four intravenous drugs we studied. Moreover, SCAMP studies at both γ 2I242C and γ 2L246C indicated no protection by these anesthetics, even at twice the concentrations that protected residues in other inter-subunit pockets. Thus, we found no evidence of intravenous anesthetic binding in the α^+ – γ^- transmembrane interfacial pocket. We tested whether the α^+ – γ^- site was accessible to a small flexible drug, n-octanol, but observed no protection. Others have suggested that ivermectin, a macrocyclic lactone that modulates GABA_A receptors, may bind in this interface⁴².

Conclusions

Our SCAMP results are in remarkable agreement with anesthetic photolabeling studies in GABA_A receptors, while identifying new drug interactions that indicate overlapping but non-congruent sites for etomidate/propofol in β^+ – α^- interfaces and mTFD-MPAB/propofol in α^+ – β^- and γ^+ – β^- interfaces. The functional characteristics of substituted tryptophan mutants appear useful for probing allosteric linkages to the channel gate, but unreliable for identifying drug binding contacts. SCAMP provided no evidence that alphaxalone binds near any of the six residues we studied. Finally, our studies in γ 2-M1 indicate coupling to channel gating similar to that in the four inter-subunit anesthetic sites, but no anesthetic contacts. Thus, the α^+ – γ^- transmembrane interface in α 1 β 3 γ 2L GABA_A receptors is a potentially unique modulator site with no established ligands; in other words, an orphan site.

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Summary Statement

Anesthetic contacts in GABA_A receptors are incompletely mapped. We showed that substituted cysteine modification-protection agrees with anesthetic photolabeling, and used this approach to test contacts between four anesthetics and all five inter-subunit transmembrane pockets.

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Figure 1. GABA_A receptor transmembrane inter-subunit anesthetic sites

The diagram depicts the arrangements of $\alpha 1$ (yellow), $\beta 3$ (blue), and $\gamma 2L$ (green) subunits and each subunit's transmembrane four-helix bundle (M1 to M4). The '+' and '-' interfacial surfaces of each subunit, corresponding respectively to M3 and M1 aspects, are identified. The approximate position of photolabeled residues, $\alpha 1M236$ and $\beta 3M227$ are labeled in magenta, while their homologs on other subunits (see Table 1) are labeled in black. We also depict the hypothesized inter-subunit sites for etomidate (red rhombi), mTFD-MPAB (*R*-5-allyl-1-methyl-5-(*m*-trifluoromethyl-diazirinyphenyl) barbituric acid; green rectangles), and propofol (white hexagons), based on photolabeling data.

Figure 2. GABA concentration-responses and anesthetic-shifts in $\alpha 1$ -M1 and $\beta 3$ -M1 tryptophan substituted GABA_A receptor mutants

The top panel shows wild-type peak current data (mean $\pm$ sem) for GABA alone (black circles), combined with 3.2 μM etomidate (red triangles), or combined with 8 μM mTFD-MPAB (*R*-5-allyl-1-methyl-5-(*m*-trifluoromethyl-diazirinyphenyl) barbituric acid; green diamonds). Normalization for control responses was to maximal GABA and to GABA plus anesthetic for anesthetic shift studies. The four other panels show similar data for two tryptophan-substituted $\alpha 1$ -M1 mutants and two $\beta 3$ -M1 mutants (labeled in each panel).

Fitted GABA EC₅₀s and EC₅₀ shifts in the presence of anesthetics are summarized in Table 2.

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Figure 3. Anesthetic EC5 enhancement in wild-type vs $\alpha 1$ -M1 and $\beta 3$ -M1 substituted tryptophan GABA_A receptor mutants

The bar-graph depicts GABA EC5 enhancement ratios (mean $\pm$ sem) for five receptor types (x-axis labels) and four equi-potent anesthetic solutions: 3.2 μ M etomidate (ETO; red), 8 μ M mTFD-MPAB (*R*-5-allyl-1-methyl-5-(*m*-trifluoromethyl-diazirinyphenyl) barbituric acid; green), 5 μ M propofol (PRO; white), and 2.5 μ M alphaxalone (ALFAX; purple). The number of oocytes studied for each interaction is indicated by the numbers in each bar. Each drug's effect in mutants was compared to the same drug effect in wild-type (by two-way ANOVA). Differs from wild-type at ** $p < 0.01$, *** $p < 0.001$.

Figure 4. Substituted cysteine modification and anesthetic protection in $\alpha 1M236C\beta 3\gamma 2L$ receptors

The bar graph summarizes pCMBS modification rate data in the presence of GABA alone and in the presence of four anesthetics (x-axis labels: ALX is alphaxalone; ETO is etomidate; MPAB is *R*-5-allyl-1-methyl-5-(*m*-trifluoromethyl-diazirinyphenyl) barbituric acid; PRO is propofol). We have previously published data showing the functional effect of pCMBS modification in this mutant and protection by etomidate and propofol ^{21,31}. Color coding by drug is the same as in Figure 3 and the number of cells studied for each condition is indicated by the numbers in each bar. This mutant is characterized by low GABA efficacy

(Table 2) and pCMBS modification in the presence of alphaxalone represents a control modification condition with high channel open probability matching that in the presence of other anesthetics ²¹. ** Differs from the rate with pCMBS + GABA + alphaxalone at $p < 0.01$ (one way ANOVA).

Figure 5. Substituted cysteine modification and anesthetic protection in $\alpha 1L232C\beta 3\gamma 2L$ receptors

Panel A shows a series of current sweeps recorded from a single oocyte expressing $\alpha 1L232C\beta 3\gamma 2L$ receptors before and after a series of 10 s exposures to p-chloromercuribenzenesulfonate (pCMBS) + GABA (arrows). Red traces show current elicited by 3 μM GABA (~EC₅₀) and the black traces show current elicited by 1 mM GABA (black bars above traces indicate GABA application). The final sweeps depict the effect of full modification by 10 μM pCMBS + GABA for 20 s (asterisk and arrow). **Panel B** shows normalized low:high GABA response ratios and linear least squares fits for individual oocytes from all control modification studies (black symbols and lines) and in the presence of 3.2 μM etomidate (red symbols and lines). **Panel C** is a bar graph summarizing $\alpha 1L232C\beta 3\gamma 2L$ modification rate data in control studies and in the presence of four anesthetics (x-axis labels: ALX is alphaxalone; ETO is etomidate; MPAB is *R*-5-allyl-1-methyl-5-(*m*-trifluoromethyl-diazirinyphenyl) barbituric acid; PRO is propofol). The number of cells studied for each condition is indicated by the numbers in each bar. *Differs from pCMBS + GABA at $p < 0.05$.

Figure 6. Substituted cysteine modification and anesthetic protection in $\alpha 1\beta 3M227C\gamma 2L$ and $\alpha 1\beta 3L231C\gamma 2L$ receptors

Panel A shows a series of traces recorded from a single oocyte expressing $\alpha 1\beta 3M227C\gamma 2L$ receptors. Currents were elicited with 2 mM GABA (black bars above traces indicate GABA applications) before and after a series of 10 s exposures to GABA + 100 μ M *p*-chloromercuribenzenesulfonate (pCMBS; arrows). The final trace was recorded after modification with GABA + 1 mM pCMBS for 20 s. **Panel B** shows normalized peak current data and linear rate analyses for cells modified with pCMBS + GABA (black symbols and lines) and cells modified with pCMBS + GABA + 8 μ M mTFD-MPAB (green symbols and lines). **Panel C** is a bar graph summarizing $\alpha 1\beta 3M227C\gamma 2L$ modification rate data in the absence and presence of four anesthetics (x-axis labels: ALX is alphaxalone; ETO is etomidate; MPAB is *R*-5-allyl-1-methyl-5-(*m*-trifluoromethyl-diazirinyphenyl) barbituric acid; PRO is propofol). **Panel D** shows a series of traces recorded from a single oocyte expressing $\alpha 1\beta 3L231C\gamma 2L$ receptors. Currents were elicited both 2 μ M GABA (red traces) and 1 mM GABA (black traces) before and after a series of 10 s exposures to GABA + 25 μ M pCMBS (arrows). Black bars above traces indicate GABA applications. The final trace was recorded after modification with GABA + 250 μ M pCMBS for 20 s. **Panel E** shows normalized low:high GABA response ratios and linear least squares rate analyses for cells modified with pCMBS + GABA (black symbols and lines) and cells modified with pCMBS + GABA + 8 μ M mTFD-MPAB (green symbols and lines). **Panel F** is a bar graph summarizing $\alpha 1\beta 3L231C\gamma 2L$ modification rate data in the absence and presence of four anesthetics (x-axis labels). In panels C and F, results statistically differing from pCMBS + GABA control are * $p < 0.05$ and ** $p < 0.01$.

Figure 7. GABA concentration-responses and anesthetic modulation of $\alpha 1\beta 3\gamma 2I242W$ and $\alpha 1\beta 3\gamma 2L246W$ receptors

Panel A shows peak current results (mean $\pm$ sem) for GABA-dependent activation of $\alpha 1\beta 3\gamma 2I242W$ receptors with GABA alone (black circles) and in the presence of 3.2 μ M etomidate (red triangles). Both data sets are normalized to 3 mM GABA responses, illustrating the low efficacy of GABA alone. Lines through data represent logistic fits (Eq. 1, methods). Fitted EC₅₀ and shift results are reported in Table 2. **Panel B** shows peak current results (mean $\pm$ sem) for GABA-dependent activation of $\alpha 1\beta 3\gamma 2L246W$ receptors with

GABA alone (black circles) and in the presence of 3.2 μ M etomidate (red triangles). Both data sets are normalized to 1 mM GABA responses. Lines through data represent logistic fits. Fitted results are reported in Table 2. **Panel C** is a bar graph summarizing GABA EC₅ enhancement results for both $\alpha 1\beta 3\gamma 2I242W$ and $\alpha 1\beta 3\gamma 2L246W$ receptors. At equipotent concentrations, none of the anesthetics (x-axis labels: ALX is alphaxalone; ETO is etomidate; MPAB is *R*-5-allyl-1-methyl-5-(*m*-trifluoromethyl-diazirinyphenyl) barbituric acid; PRO is propofol) differ in their modulation of these two mutant receptors.

Figure 8. Substituted cysteine modification and anesthetic protection in $\alpha 1\beta 3\gamma 2I242C$ and $\alpha 1\beta 3\gamma 2L246C$ receptors

Panel A shows a series of traces recorded from a single oocyte expressing $\alpha 1\beta 3\gamma 2I242C$ receptors. Currents were elicited with 3.5 μM GABA (red traces) and 1 mM GABA (black traces) before and after a series of exposures to GABA + 10 μM *p*-chloromercuribenzenesulfonate (pCMBS; arrows). Black bars above traces indicate GABA applications. The final trace was recorded after modification with GABA + 100 μM pCMBS for 20 s. **Panel B** shows normalized low:high GABA response ratios and linear least squares rate analyses for cells modified with pCMBS + GABA. **Panel C** is a bar graph summarizing $\alpha 1\beta 3\gamma 2I242C$ modification rate data in the absence and presence of four anesthetics (x-axis labels: ALX is alphaxalone; ETO is etomidate; MPAB is *R*-5-allyl-1-methyl-5-(*m*-trifluoromethyl-diazirinyphenyl) barbituric acid; PRO is propofol). **Panel D** shows a series of traces recorded from a single oocyte expressing $\alpha 1\beta 3\gamma 2L246C$ receptors. Currents were elicited both 1.5 μM GABA (red traces) and 1 mM GABA (black traces) before and after a series of exposures to GABA + 500 μM pCMBS (arrows). Black bars above traces indicate GABA applications. The final trace was recorded after modification with GABA + 1 mM pCMBS for 60 s. **Panel E** shows normalized low:high GABA response ratios and linear least squares rate analyses for cells modified with pCMBS + GABA. **Panel F** is a bar graph summarizing $\alpha 1\beta 3\gamma 2L246C$ modification rate data in the absence and presence of four anesthetics (x-axis labels). None of the rates with anesthetics differed significantly from control.

GABA_A Receptor M1 Helix Amino Acid Sequence Alignments

Table 1

	Position after Conserved GYF																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
α1	G224	Y	F				P	C	I	M236	T	V	I	L	S	Q	V	S	F	W	L	N	R	E
β3	G219	Y	F				P	S	I	L231	I	T	I	L	S	W	V	S	F	W	I	N	Y	D
γ2	G234	Y	F				P	C	T	L246	I	V	V	L	S	W	V	S	F	W	I	N	K	D

The aligned amino acid single-letter code sequences are from human genomic data for GABA_A receptor α1 (UniProtKB P14867), β3 (UniProtKB P28472) and γ2 (UniProtKB P18507). The conserved pre-M1 GYF sequence is highlighted in pink. The two highlighted residues α1M236 and β3M227 have been photolabeled by derivatives of intravenous anesthetics.

Table 2
Functional and Pharmacological Characteristics of $\alpha 1\beta 3\gamma 2L$ GABA_A Receptors with M1 Tryptophan Substitutions

Receptor Type	GABA EC ₅₀ (μ M) [95% CI] (n)	GABA Efficacy mean $\pm$ se (n)	Spont. Activation mean $\pm$ se (n)	Etomidate-induced GABA EC ₅₀ Shift [95% CI] (n)	MPAB-induced GABA EC ₅₀ Shift [95% CI] (n)
$\alpha 1\beta 3\gamma 2L$	18 [16 to 20] (14)	0.88 $\pm$ 0.025 (5)	<0.005 (5)	16 [13 to 20] (5)	12 [9.7 to 15] (4)
$\alpha 1L232W\beta 3\gamma 2L$	4.8 [3.6 to 6.4] * (3)	0.69 $\pm$ 0.036 ** (3)	<0.005 (4)	2.2 [1.6 to 3.1] * (3)	7.4 [4.6 to 12.0] (3)
$\alpha 1M236W\beta 3\gamma 2L$	1.5 [1.1 to 2.3] * (3)	0.97 $\pm$ 0.011 ** (6)	0.04 $\pm$ 0.014 * (4)	1.6 [1.0 to 2.6] * (3)	2.4 [1.4 to 4.4] * (3)
$\alpha 1\beta 3M227W\gamma 2L$	38 [30 to 47] * (3)	0.99 $\pm$ 0.013 ** (3)	<0.005 (4)	12 [9.2 to 15] (3)	4.8 [3.8 to 6.2] * (3)
$\alpha 1\beta 3L231W\gamma 2L$	4.2 [3.7 to 4.8] * (3)	0.91 $\pm$ 0.023 (3)	0.035 $\pm$ 0.0045 * (4)	4.4 [3.1 to 6.3] * (3)	7.8 [5.0 to 12] (3)
$\alpha 1\beta 3\gamma 2I242W$	105 [91 to 121] * (3)	0.75 $\pm$ 0.042 ** (4)	<0.005 (4)	15 [12 to 19] (3)	–
$\alpha 1\beta 3\gamma 2L246W$	5.3 [3.6 to 7.7] * (5)	0.97 $\pm$ 0.011 * (3)	0.11 $\pm$ 0.017 ** (5)	4.9 [2.7 to 8.8] * (7)	–

MPAB is *R*-5-allyl-1-methyl-5-(*m*-trifluoromethyl-diazirinylophenyl) barbituric acid.

Differs from wild-type at: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 3
Functional and Pharmacological Characteristics of $\alpha 1\beta 3\gamma 2L$ GABA_A Receptors with M1 Cysteine Substitutions

Receptor Type	GABA EC ₅₀ (μ M) [95% CI] (n)	GABA Efficacy mean $\pm$ se (n)	Spont. Activation mean $\pm$ se (n)	GABA EC ₅ Fold-Enhancement ^a mean [range] (n)	Effect of pCMBS Modification
$\alpha 1L232C\beta 3\gamma 2L$ ^b	77 [*] [56 to 107] (5)	0.95 $\pm$ 0.03 ^{**} (5)	<0.005 (4)	9.8 [8.1 to 11.4] (12)	Increase low:high GABA response ratio
$\alpha 1M236C\beta 3\gamma 2L$ ^b	360 [*] [260 to 510] [*] (6)	0.24 $\pm$ 0.06 ^{***} (6)	0.04 $\pm$ 0.014 ^{**} (4)	10.6 [8.6 to 12] (15)	Increase low:high GABA response ratio
$\alpha 1\beta 3M227C\gamma 2L$	29 [*] [25 to 33] (6)	0.65 $\pm$ 0.050 [*] (4)	<0.005 (4)	10.1 [7.4 to 14.3] (12)	Reduce high GABA response
$\alpha 1\beta 3L231C\gamma 2L$	53 [*] [39 to 71] (3)	0.66 $\pm$ 0.031 ^{**} (3)	<0.005 (4)	9.3 [5.8 to 12] (12)	Increase low:high GABA response ratio
$\alpha 1\beta 3\gamma 2I242C$	16 [13 to 19] (3)	0.94 $\pm$ 0.033 [*] (4)	<0.005 (4)	9.3 [5.6 to 11.4] (12)	Increase low:high GABA response ratio
$\alpha 1\beta 3\gamma 2L246C$	14 [12 to 17] (6)	0.86 $\pm$ 0.034 (4)	<0.005 (4)	12.0 [8.7 to 15] (12)	Increase low:high GABA response ratio

pCMBS is *p*-chloromercuribenzenesulfonate.

Differs from wild-type (see Table 2) at: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

^a GABA EC₅ Enhancement results are for all four study drugs at $2 \times$ EC₅₀ for tadpole LoRR. Each drug was tested in at least 3 oocytes.

^b GABA EC₅₀, efficacy and spontaneous activation results are from Stewart et al ²¹.

Table 4

Photolabeling^a vs. Substituted Tryptophan Sensitivity^b and SCAMP^c in $\alpha 1\beta 3\gamma 2L$ GABA_A Receptor M1 Domains

Drug Residue	Etomidate		mTFD-MPAB		Propofol		Alphaxalone	
	W	PL	C	W	PL	C	W	PL
$\alpha 1L232$	+	-	+	-	-	+	-	ND
$\alpha 1M236$	+	+	+	-	-	+	+	-
$\beta 3M227$	-	-	-	+	+	+	-	-
$\beta 3L231$	+	-	-	-	-	-	-	-
$\gamma 2L242$	-	-	-	-	-	ND	-	ND
$\gamma 2L246$	-	-	-	-	-	ND	-	ND

^aPhotolabeling at $\alpha 1M236$ and $\beta 3M227$ (highlighted rows) is indicated by a bolded '+' in the middle column (PL) under each anesthetic if the specified residue was an incorporation site for anesthetic analogs: R-(+)-azi-etomidate, R(-)-mTFD-MPAB, *m*-azi-propofol, or 6-azi-pregnanalane. The negative result for $\alpha 1M236$ -alphaxalone was inferred from photolabeling competition using azietomidate. 'ND' indicates an absence of direct or indirect photolabeling data, due to missing subunits in the photolabeled receptors.

^bIn the left column (W) under each drug, '+' indicates that a Trp substitution significantly reduces enhancement of GABA EC₅₀ responses by the anesthetic (see Figs 3 and 7).

^cSCAMP is substituted cysteine modification-protection. In the right column (C) under each anesthetic, '+' indicates that the drug significantly inhibited the rate of covalent modification at the cysteine-substituted sidechain (see Figs 4–6 and 8).