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A Cysteine Substitution Probes β3H267 Interactions with Propofol and Other Potent Anesthetics in α1β3γ2L Gamma-Aminobutyric Acid Type A Receptors

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Abstract

Background—Anesthetic contact residues in γ-aminobutyric acid type A ($GABA_A$) receptors have been identified using photolabels, including two propofol derivatives. O-propofol-diazirine labels H267 in β3 and α1β3 receptors, while m-azi-propofol labels other residues in intersubunit clefts of α1β3. Neither label has been studied in αβγ receptors, the most common isoform in mammalian brain. In αβγ receptors, other anesthetic derivatives photolabel m-azi-propofol labeled residues, but not βH267. Our structural homology model of α1β3γ2L receptors suggests that β3H267 may abut some of these sites.

Methods—Substituted cysteine modification-protection was used to test β3H267C interactions with four potent anesthetics: propofol, etomidate, alphaxalone, and *R*-5-allyl-1-methyl-5-(*m*-trifluoromethyl-diazirinylphenyl) barbituric acid (mTFD-MPAB). We expressed α1β3γ2L or α1β3H267Cγ2L $GABA_A$ receptors in *Xenopus* oocytes. We used voltage clamp electrophysiology to assess receptor sensitivity to GABA and anesthetics, and to compare *para*-chloromercuribenzenesulfonate (pCMBS) modification rates with GABA *versus* GABA plus anesthetics.

Results—Enhancement of GABA EC5 responses by equi-hypnotic concentrations of all four anesthetics was similar in α1β3γ2L and α1β3H267Cγ2L receptors ($n \geq 3$). Direct activation of α1β3H267Cγ2L receptors, but not α1β3γ2L, by mTFD-MPAB and propofol was significantly greater than the other anesthetics. Modification of β3H267C by pCMBS ($n \geq 4$) was rapid and accelerated by GABA. Only mTFD-MPAB slowed β3H267C modification (~2-fold; $p = 0.011$).

Conclusions—β3H267 in α1β3γ2L $GABA_A$ receptors contacts mTFD-MPAB, but not propofol. Our results suggest that β3H267 is near the periphery of one or both transmembrane inter-subunit (α+/β− and γ+/β−) pockets where both mTFD-MPAB and propofol bind.

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Introduction

Propofol, etomidate, barbiturates, and alphaxalone enhance gamma-aminobutyric acid type A ($GABA_A$) receptor gating, contributing to sedation, hypnosis, and immobilization [1–3]. $GABA_A$ receptors are pentameric ligand-gated ion channels (pLGICs). The most common subtypes in mammalian brain contain two α, two β, and one γ subunit arranged as in Figure 1 [4,5]. Each subunit has an N-terminal extracellular domain and a four-helix (M1 to M4) transmembrane domain (TMD). Subunit interfacial surfaces are designated “plus; +” (M3 side) or “minus; −” (M1 side) [4]. Current structural homology models of αβγ receptors, based on crystallized homomeric pLGICs from bacteria, nematodes, and humans β3, are similar [6–11].

Anesthetic binding residues in $GABA_A$ receptors (Fig 1C, D) have been identified using both photolabel derivatives (Fig 2, Table 1) and substituted cysteine modification-protection (Table 1). Two propofol derivatives, m-azi-propofol (azi-Pm) and o-propofol diazirine (o-PD), photolabel distinct residues [12,13]. In α1β3 receptors, azi-Pm labels residues in β3-M3 (β3M286), α1-M1 (α1M236), and β3-M1 (β3M227) [12]. These residues are also labeled in αβγ receptors by either azi-etomidate or the potent barbiturate *R*-5-allyl-1-methyl-5-(*m*-trifluoromethyl-diazirinylphenyl) barbituric acid (mTFD-MPAB) (Fig 1C, D and Table 1) [14,15]. Propofol inhibits photolabeling by azi-Pm, azi-etomidate, or mTFD-MPAB [12,15,16]. *O*-PD also inhibits azi-etomidate and mTFD-MPAB incorporation [12]. However, in β3 homomers and α1β3 receptors o-PD uniquely labels β3H267 (M2–17′), which is not labeled by other anesthetics [13] (Table 1). To date, neither azi-Pm nor o-PD has been studied in αβγ $GABA_A$ receptors.

Conflicting structural interpretations of propofol photolabeling results, and particularly the role of βH267, emerge from homology model analyses. *In silico* docking calculations for propofol in the β3 crystal structure suggest that H267 contributes to binding sites separate from those where azi-Pm binds [17]. In contrast, our α1β3γ2L homology model (Fig. 1) locates β3H267 near and possibly within α+/β− and γ+/β− pockets containing residues labeled by both azi-Pm and mTFD-MPAB.

Substituted cysteine modification-protection is sensitive to steric interactions between anesthetics and putative contact residues. Sulfhydryl-specific reagents covalently modify accessible cysteine-substituted residues, usually producing functional changes [18]. Bound anesthetic may hinder chemical modification of cysteines located near or within anesthetic sites. For example, both etomidate and propofol block modification of αM236C and βM286C in α1β2/3γ2 receptors [19–21] (Table 1). This approach also has identified several non-photolabeled anesthetic contact residues in β+/α− interfaces (Fig 1, Table 1) [20,22,23], but has not been reported for anesthetic interactions with other transmembrane interfacial pockets.

In the current study, we tested the hypothesis that in α1β3γ2L receptors β3H267 is near propofol and mTFD-MPAB sites in α+/β− and γ+/β− interfaces, but not those for etomidate or alphaxalone in β+/α− interfaces [24,25]. Using voltage-clamp electrophysiology we pharmacologically characterized α1β3H267Cγ2L receptors and compared rates of β3H267C

modification by *para*-chloromercuribenzesulfonate (pCMBS) in the absence vs. presence of anesthetics. The β3H267C mutation selectively sensitized α1β3γ2L to direct activation by propofol and mTFD-MPAB. Modification of β3H267C by pCMBS was rapid, enhanced by GABA, and slowed by mTFD-MPAB, but not other anesthetics. We infer that β3H267 is located in or near mTFD-MPAB binding sites in α1β3γ2L receptors.

Materials and Methods

Animal use

Female *Xenopus laevis* were used with approval from the Massachusetts General Hospital Institutional Animal Care & Use Committee. Frogs were housed in a veterinary-supervised environment in accordance with local and federal guidelines. Frogs were anesthetized by immersion in 0.2% tricaine (Sigma-Aldrich, St. Louis, MO) prior to mini-laparotomy to harvest oocytes.

Chemicals

R(+)-Etomidate was obtained from Bedford Laboratories (Bedford, OH). The clinical preparation in 35% propylene glycol was diluted directly into buffer. Propylene glycol at the resulting concentrations has no effect on $GABA_A$ receptor function [26]. Propofol was purchased from Sigma Aldrich (St. Louis, MO) and alphaxalone was purchased from MP Biomedical (Solon, OH). Both propofol and alphaxalone were prepared as stock solutions in dimethylsufoxide. After dilution into electrophysiology buffer, dimethylsufoxideconcentrations were below 0.1%, and produced no effects on either wild-type or mutant $GABA_A$ receptors. R-mTFD-MPAB was a gift from Dr. Karol Bruzik, Ph.D. (Dept. of Medicinal Chemistry, Univ. Illinois Chicago, IL) and prepared as a 100 mM stock in methanol. After dilution for electrophysiology studies, methanol was below 0.01%, which produced no significant modulation of either wild-type or mutant $GABA_A$ receptors. Picrotoxin (PTX) was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved (2 mM) in electrophysiology buffer. *p*-Chloromercuribenzenesulfonic acid sodium salt (pCMBS) was purchased from Toronto Research Chemicals (North York, Ontario, Canada). All other chemicals were purchased from Sigma-Aldrich.

Molecular Biology

Complementary DNAs for human $GABA_A$ receptor α1, β3, and γ2L subunits were cloned into pCDNA3.1 vectors (Invitrogen, Carlsbad, CA). A mutation encoding β3H267C was created with oligonucleotide-directed mutagenesis, using a QuikChange kit (Agilent Technologies, Santa Clara, CA). Several clones from the mutagenesis reaction were subjected to DNA sequencing through the entire β3 coding region to confirm the presence of the intended mutation and absence of stray mutations. A single mutant clone was selected for further use.

Oocyte Electrophysiology

Messenger RNA synthesis and *Xenopus* oocyte expression were performed as we have described [27]. Electrophysiology experiments were conducted at room temperature (21–23 °C). Oocytes were voltage-clamped at −50 millivolts and signals were low-pass filtered at 1

kiloHertz (Model OC-725B, Warner Instruments, Hamden, CT). Electrophysiological signals were digitized at 200 Hertz (iWorx RA834, iWorx Systems Inc, Dover, NH) and recorded digitally on a personal computer running Labscribe v3 software (iWorx Systems Inc.). Oocyte superfusion in a custom-build flow chamber was software-controlled through the iWorx RA834 interface to solenoid switches (ALA-VM8, ALA Scientific Associates, Farmingdale, NY) and a sub-microliter dead-volume manifold. Five-fold data reduction and further low-pass (10 Hertz) digital filtering (using Clampfit 9.0, Molecular Devices, Sunnyvale, CA) were used in preparing traces for display in figures.

Electrophysiology solutions, including those containing GABA and/or anesthetics were based on ND96 (in mM: 96 NaCl, 2 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, pH 7.5). Peak current responses to GABA concentrations ranging from 0.1 μM to 3 mM, alone or co-applied with anesthetics, were assessed in *Xenopus* oocytes ($n \geq 3$ from at least two frogs) using two microelectrode voltage clamp electrophysiology[28]. GABA applications varied in duration, depending on the time to reach steady-state peak current. Normalizing GABA responses at maximal GABA (1 mM), were recorded every 2nd or 3rd sweep. Picrotoxin-sensitive leak was measured using 2 mM PTX, followed by >5 minute washout and a maximal GABA response test. Propofol (5 μM) or alphaxalone (2 μM) were used as gating enhancers together with maximal GABA to assess GABA efficacy[29]. Direct activation and GABA enhancement were assessed in both wild-type and α1β3H267Cγ2L receptors using equi-potent anesthetic concentrations (2 × EC50 for loss of righting reflexes in *Xenopus* tadpoles = 2.5 μM alphaxalone, 5 μM propofol, 3.2 μM etomidate, and 8 μM mTFD-MPAB). The EC5 GABA concentration was identified for individual oocytes by testing GABA concentrations ranging from 2 to 4 μM. After establishing stable EC5 and 1 mM responses, oocyte currents were recorded during exposure to first anesthetic alone for 30s, followed by anesthetic combined with EC5 GABA for another 15 to 30s.

Electrophysiological Data Analysis

Analyses for agonist concentration-responses, and propofol-induced left shift followed our approach described elsewhere [27,29]. Peak GABA-stimulated currents were normalized to maximal GABA responses, and GABA concentration-response data for individual oocytes in the absence and presence of propofol were fitted with logistic functions using non-linear least squares (Graphpad Prism v.5):

$$\mathbf{I}_{\mathrm{Agonist}} = \frac{\mathbf{I}_{\max} - \mathbf{I}_{\min}}{1 + 10^{(\log \mathbf{EC}50 - \log[\mathbf{Agonist}]) * \mathbf{nH}}} + \mathbf{I}_{\min} \quad \text{Eq. 1}$$

where EC_{50} is the half-maximal activating concentration and nH is Hill slope.

EC_{50} shift ratio was calculated from the difference in log(GABA EC_{50}) values [$\Delta\log(EC_{50})$] measured in the presence of 5 μM propofol versus control.

Cysteine Modification with pCMBS

Voltage-clamped oocytes expressing $GABA_A$ receptors were repetitively activated with alternating EC5 and 1 mM GABA pulses every five minutes until at least three sequential sets of responses were constant (± 5%). Oocytes were then exposed to pCMBS (alone, with

GABA, or with GABA + anesthetic) for 5 to 12s followed by 5 min ND96 wash. In oocytes expressing wild-type α1β3γ2L receptors, exposure to pCMBS (1 mM x 60 s, followed by a 5 minute wash in ND96 buffer) produced no significant changes in currents stimulated with low (EC5 = 4 μM) or 1 mM GABA. We tested a range of pCMBS concentrations on oocytes expressing α1β3H267Cγ2L receptors. Exposure to 1 μM pCMBS for 10 s resulted in an approximately 5-fold increase in response to low GABA (EC5 = 3 μM) relative to saturating GABA (1 mM). In most oocytes, the change in response ratio ($I_{3\mu M}/I_{max}$) was associated with increased response to 3 μM GABA and a modest reduction in response to 1 mM GABA. Repeated 10s exposures to 1 μM pCMBS did not produce further change in response ratio, suggesting that β3H267C modification was complete after a single exposure. For experiments comparing the apparent initial covalent modification rates in α1β3H267Cγ2L receptors, we used a much lower pCMBS concentration of 10 nM. Two or three 5 to 12 s applications of 10 nM pCMBS (each followed by 5 min ND96 wash) typically resulted in less than a doubling of $I_{3\mu M}/I_{max}$, i.e. less than 20% of the change associated with complete modification. After repeated exposures to 10 nM pCMBS, each oocyte was also exposed to 1 μM pCMBS for 10s to assess I_{EC5}/I_{max} following full modification.

To test for anesthetic protection (inhibition of β3H267C modification), apparent modification rates with pCMBS plus 1 mM GABA were compared to rates with pCMBS plus 1 mM GABA and anesthetic. The GABA-bound receptor was chosen as the index condition, because GABA binding enhances the affinity of receptors for anesthetics, thereby increasing anesthetic site occupancy [21]. The anesthetic concentrations used in protection studies were 10 μM alphaxalone, 10 and 30 μM etomidate, 5, 10, and 30 μM propofol, and 8 and 16 μM mTFD-MPAB. These anesthetic concentrations enhance activation of both wild-type and mutant $GABA_A$ receptors at least 10-fold (see results), and estimates of etomidate[26] and propofol[30] affinities for GABA-bound receptors suggest that over 90% of anesthetic sites are occupied under these conditions. For modification rate analysis, $I_{3\mu M}/I_{max}$ response ratios were normalized to the pre -modification control, and plotted against cumulative pCMBS exposure in units of nM × sec. Normalized response ratios were fitted by linear least squares to determine the apparent initial modification rate (slope, in $M^{-1}s^{-1}$). We fitted modification rates for both individual oocytes and for combined response ratio data from groups of oocytes for each condition. These resulted in slightly different mean and standard error values, due to differential data weighting, without affecting our overall conclusions.

Molecular Structural Modeling

We used a structural model for the α1β3γ2 $GABA_A$ receptor based on GluCl bound to ivermectin (PDB 3RHW)[10], which we have described in a prior publication [22]. The optimized structure was visualized and analyzed using University of California San Francisco Chimera v1.10. Optimized molecular structure models for the anesthetic drugs were built and analyzed using Avogadro v1.1.1 [31].

Statistical Analysis

Oocytes were obtained from at least two frogs and randomly selected for each experiment. Blinding was not used during experiments or analysis. Group sizes ($n \geq 3$ for functional characterization; $n \geq 4$ for modification rate comparisons) were based on prior experience with these techniques. Additional control modification experiments (with GABA plus pCMBS) were performed with each set of protection studies. Results are reported as mean ± standard error unless otherwise noted. Statistical analyses were performed using Prism 5.02 (Graphpad Software, Inc., La Jolla, CA). Statistical comparisons of anesthetic direct activation and GABA enhancement in both wild-type and α1β3H267Cγ2L receptors was based on two-way ANOVA and pairwise Bonferroni post-tests. Apparent pCMBS modification rates measured under multiple conditions (i.e. sets of individual oocyte results) were compared using Kruskal-Wallis with Dunn's Multiple Comparison test. Other pairwise comparisons were performed using Student's t-tests or Mann-Whitney. Statistical significance was inferred at $p < 0.05$.

Results

Xenopus oocytes injected with messenger RNA mixtures encoding α1, wt β3 or β3H267C, and γ2L $GABA_A$ receptor subunits were studied using two-electrode voltage-clamp. In wild-type control experiments, α1β3γ2L receptors produced GABA-dependent currents with EC_{50} averaging 31 μM (data not shown; n = 3; 95% CI = 18 to 49 μM), consistent with previous reports [26]. Propofol (5 μM) produced a 12-fol GABA EC_{50} shift in wild-type receptors (data not shown; n = 3, 95% CI = 6.3 to 23-fold).

Voltage-clamped oocytes expressing α1β3H267Cγ2L receptors produced inward currents in response to GABA, in a concentration-dependent and reversible manner (Fig. 3A). The fitted GABA EC_{50} value for α1β3H267Cγ2L receptors was 25 μM (n = 3; 95% CI = 19 to 32 μM), similar to wild-type. Co-application of GABA with propofol (5 μM) enhanced currents elicited by GABA concentrations below 100 μM (Fig 3B), producing a 15-fold (95% CI = 7.7 to 30-fold) leftward shift in the averaged concentration-response curve (Fig 3C) to 1.6 μM (n = 3; 95% CI = 0.83 to 3.2 μM). Again, this result does not significantly differ from wild-type, indicating that mutant receptors retain near-normal sensitivity to propofol. In oocytes expressing α1β3H267Cγ2L receptors with maximal peak currents over 5 μA, picrotoxin (2 mM) applied in the absence of GABA did not alter basal leak currents (Fig. 3D; n = 3), indicating that spontaneous receptor activation is below the detection threshold (about 5 nA or 0.1% of maximal peak). Currents elicited with 1 mM GABA were not enhanced by propofol, indicating that high GABA concentrations activated nearly 100% of α1β3H267γ2L receptors (Fig 3D). We have previously estimated that spontaneous activation of wild-type receptors has a probability below 0.01% and that maximal GABA efficacy in wild-type receptors is approximately 85% [26].

We extended our study of anesthetic interactions at β3H267 to three other potent anesthetics that modulate $GABA_A$ receptors: etomidate, alphaxalone, and mTFD-MPAB. Using voltage-clamp electrophysiology, we compared the effects of equipotent drug concentrations (2 × the EC_{50} for loss-of-righting-reflexes in tadpoles) in both wild-type α1β3γ2L (Fig. 4A) and α1β3H267Cγ2L (Fig. 4B) $GABA_A$ receptors. In current recordings where oocytes were

first exposed to anesthetic for 30 s followed by anesthetic + EC5 GABA, we found that 5 μM propofol, 3.2 μM etomidate, 2.5 μM alphaxalone, and 8 μM mTFD-MPAB produce indistinguishable (~ 10-fold) enhancing effects on EC5 GABA responses in both α1β3γ2L and α1β3H267Cγ2L receptors (Fig 4C). These studies also revealed that both propofol and mTFD-MPAB directly activated α1β3H267Cγ2L receptors significantly more than the other anesthetics, and also far more than these drugs activated wild-type receptors (Fig 4D).

After applying pCMBS (1 μM for 10 s) to voltage-clamped oocytes expressing α1β3H267Cγ2L receptors, followed by 5 min wash in electrophysiology buffer, we observed a 5-fold increase in the response to 3 μM GABA (approximate EC5) relative to the 1 mM GABA response (an example is shown in Fig 5A). Repeated exposure to 1 μM pCMBS (with post-exposure wash) did not further increase the normalized response ratio ($I_{3\mu M}/I_{1mM}$), indicating that a single 10 s exposure fully and irreversibly modified all receptors. In contrast, when oocytes expressing α1β3γ2L receptors were exposed to 1 mM pCMBS for up to 60 s, no changes were observed in spontaneous leak or current responses to low and high GABA (n = 3; not shown). Therefore, the effect of pCMBS on α1β3H267Cγ2L function was due to covalent bond formation at β3H267C.

In oocytes expressing α1β3H267Cγ2L receptors, initial pCMBS modification rates were assessed using repeated 5 to 12 s exposures to 10 nM pCMBS. At this concentration, the $I_{3\mu M}/I_{1mM}$ response ratio increased by about 40% after a cumulative 30 s of exposure (Fig 5B). Linear fits to the normalized response ratios plotted against cumulative pCMBS exposure for all oocytes (Fig 5B; n = 5) indicated an apparent slope of (mean ± se) 1.3 ± 0.19 × 10^6 M^{-1} s^{-1}. The average of individual oocyte modification rates (mean ± sem) was similar (1.3 ± 0.24 × 10^6 M^{-1} s^{-1}). When pCMBS was co-applied with 1 mM GABA (e.g. Fig. 5C), the apparent rate of modification (all oocytes; n = 9) increased to 3.6 ± 0.25 × 10^6 M^{-1} s^{-1} (Fig 5D). The average of individual oocyte modification rates with GABA was 3.9 ± 0.58 × 10^6 M^{-1} s^{-1}, three-fold higher (p = 0.0078; Mann-Whitney test) than the rate without GABA. The maximal change in normalized response ratio remained approximately 5-fold after co-application of 1 μM pCMBS with GABA (Fig 5D). Co-application of pCMBS with 1 mM GABA plus 10 μM propofol (e.g. Fig. 5E) resulted in an apparent rate of modification (all oocytes; n = 5) of 3.0 ± 0.47 × 10^6 M^{-1} s^{-1} (Fig 5F). The individual oocyte modification rates with GABA + propofol (3.5 ± 0.69 × 10^6 M^{-1} s^{-1}) and the overall effect of modification were similar to those in the presence of GABA alone. Additional protection experiments using 30 μM propofol (not shown; n = 5) also indicated no reduction in the modification rate.

We also tested whether etomidate, alphaxalone, or mTFD-MPAB alter the rate of pCMBS modification in GABA-activated α1β3H267Cγ2L receptors, applying the same approach used for propofol. Etomidate (10 and 30 μM) and alphaxalone (10 μM) produced no changes, whereas mTFD-MPAB (8 μM; Fig 5G and 5H) reduced the average modification rate approximately 2-fold (Fig. 6; p = 0.011; Mann-Whitney test). Attempts to study protection using higher (16 μM) mTFD-MPAB concentrations were complicated by very slow drug washout producing residual direct activation and desensitization of α1β3H267Cγ2L receptors, resulting in widely varying apparent modification rates in repeated experiments.

Discussion

In a α1β3γ2L background, we investigated β3H267C effects on anesthetic sensitivity, and tested whether bound anesthetics protect this cysteine from modification by pCMBS. Although βH267 was photolabeled by o-PD in β3 and α1β3 receptors, we found that in α1β3γ2L, propofol did not protect β3H267C from chemical modification. In similar studies with etomidate, alphaxalone, and mTFD-MPAB, only mTFD-MPAB reduced the rate of β3H267C modification. This suggests that β3H267 is near at least one of the two mTFD-MPAB "β−" sites in α1β3γ2L, as predicted by our structural homology model (Fig 1D) [12,15]. Our negative β3H267C protection results with etomidate and alphaxalone are also consistent with prior evidence that these anesthetics bind in β+/α− interfaces [14,24,25,32].

Earlier studies showed that βH267 mutations influence $GABA_A$ receptor modulation by both Zn^{2+} and protons [33–35]. We also found that the β3H267C mutation selectively sensitized receptors to activation by both mTFD-MPAB and propofol, linking β3H267 to channel gating and the nearby α+/β− and γ+/β− sites where these anesthetics bind. The absence of β3H267C effects on receptor agonism by GABA, etomidate, and alphaxalone rules out global allosteric effects of the mutation. Indeed, the anesthetic specificity of both pharmacological effects (Fig 4) and biochemical protection (Fig 6) indicate local interactions of β3H267 with the "β−" anesthetic sites.

Consistent with our observations, a prior study of α1β1H267Cγ2 also reported enhanced channel gating after pCMBS modification [36]. The pCMBS modification rate at β3H267C (~4×10^6 $M^{-1}s^{-1}$ with GABA) was about 10-fold faster than other TMD cysteine substitutions we have examined [20–22]. The rapid modification of β3H267C indicates a relatively high degree of probe and water exposure for a TMD sidechain,[18] but remains far slower than pCMBS reactions with free cysteine in bulk water at pH 7.5 (estimated near 10^8 $M^{-1}s^{-1}$) [37]. GABA increased the rate of modification, indicating GABA-dependent structural rearrangements near β3H267. The dynamic structural changes in the $GABA_A$ receptor TMD that accompany channel activation and desensitization remain uncertain, although comparisons of crystallized GluCl structures[9,10] and biophysical studies of bacterial pLGICs [38] in different states suggest that the extracellular ends of M2 and M3 helices tilt away from the pore, possibly expanding inter-subunit pockets and their water content.

The interpretation of our new results must consider limitations of photolabeling, cysteine modification-protection, and structural models of heteromeric $GABA_A$ receptors. Photolabeling is an unbiased method for identifying ligand contact loci. Photolabels must be structurally and pharmacologically similar to the "parent" drug of interest. Also required are sufficient target protein quantity and purity, efficient and stable photo-adduct formation, and a sensitive method for identifying incorporation sites. Limitations include the potential for photolabeling sites other than those where the parent drug acts, and for selective photochemical reactions with amino acids that may not exist in drug binding sites. The β3H267 residue was identified as the sole contact in β3 and α1β3 receptors photolabeled with o-PD using mass spectroscopic proteonomic analysis[13]. Subsequently, Jayakar et al reported that o-PD displaced azi-etomidate and mTFD-MPAB labeling in α1β3 receptors[12],

implying that o-PD interacts with residues other than β3H267 in heteromeric receptors (that contain a β3-β3 interface). Thus, o-PD photolabeling may have missed other contact residues due to technical limitations. Photolabeling results for azi-Pm and o-PD may also reflect different orientations of photo-reactive groups at ring positions 2 and 6 (presumably near βH267) relative to positions 3 and 5 when bound in the same site with steric constraints. Indeed, modifications at these propofol ring positions also produce distinct effects on drug potency/efficacy [39]. Similarly, etomidate's photolabel derivatives[14,32] have identified only a portion of its currently known contact residues. Others were identified in αβγ receptors using cysteine modification-protection.

The substituted cysteine modification-protection strategy uses an unmodified ligand and sulfhydryl-selective chemistry to test interactions at putative contact residues. Important considerations for this method include: 1) that ligand binding is retained in the cysteine-substituted mutant receptor, 2) that ligand occupies a large fraction of its sites during protection experiments, and 3) that a similar mixture of receptor states is present during modification in both the absence and presence of ligand. In our current experiments, evidence indicates that all these conditions were met. Modulation of α1β3H267Cγ2L receptors by propofol and the other anesthetics was similar to that in wild-type $GABA_A$ receptors (Fig 4C), indicating minimal changes in affinity/binding. By using high GABA, we established conditions where nearly all α1β3H267Cγ2L receptors were either in open or desensitized states that have high anesthetic affinity relative to resting/closed receptors. Propofol modestly slows $GABA_A$ receptor desensitization without altering its extent [40], implying that both open and desensitized receptors bind propofol with similar affinities. Thus, similar receptor state mixtures were present during modification with or without anesthetics. Our prior estimate of the propofol dissociation constant for GABA-bound α1β2γ2L receptors ($K_P \times d \approx 2$ μM)[30], suggests that 10 μM propofol occupies ~83% of sites and 30 μM PRO occupies ~93% of sites. Photolabeling inhibition also indicates that propofol binds to both etomidate and mTFD-MPAB sites with similar affinities [15].

We studied propofol interactions with β3H267 in α1β3γ2L, and our results do not address β3 and α1β3 receptors that were photolabeled with o-PD[13]. Propofol contact might occur only within β/β interfaces that are absent in αβγ receptors. Even if propofol contacts β3H267 in wild-type receptors, the histidine-to-cysteine mutation reduces sidechain size and may also alter orientation, reducing contact in the mutant. Given that in β3 homomers H267 is positioned between the inter-subunit cleft and the ion channel[6], it is conceivable that propofol binds near β3H267C but does not effectively protect the sulfhydryl group from pCMBS in the receptor pore. However, our "positive control" finding that mTFD-MPAB protects β3H267C indicates that this is unlikely and that the technique worked as intended. Moreover, a recent study of β3H267W effects in β3 and α1β3 also found no evidence for propofol interactions with this residue [41].

Photolabeling has established that in α1β3 receptors, propofol, azi-Pm, o-PD and mTFD-MPAB compete for binding sites in α+/β− and β+/β− interfaces[12,15]. Considering these data together with our current results suggests that β3H267 is located near the periphery of at least one of the mTFD-MPAB sites in α1β3γ2L and further from sub-regions of the β− pockets that interact with both mTFD-MPAB and propofol. In our structural homology

model, contiguous cavities extend from β3H267 to residues photolabeled by mTFD-MPAB and azi-Pm (Fig. 7A, B), including α1S270, another residue thought to interact with anesthetics [42,43]. The model-derived distances from the β3H267 imidazole to α1S270, α1A291, and α1Y294 range from 7.0 to 12.7 angstroms while from β3H267 to γ2S301 is 12.8 Å. The largest projection length of R-mTFD-MPAB is 10.9 Å, while that of propofol is 7.6 Å. Thus, mTFD-MPAB is large enough to bind near α1A291 or γ2S301 and impede pCMBS access to β3H267C, while propofol is smaller and may fail to obstruct this interaction. To fully reconcile photolabeling with our protection results we also posit that azi-Pm and o-PD both occupy β− sites overlapping those for propofol and mTFD-MPAB, yet photolabel different residues because of constrained binding orientations.

Some alternative $GABA_A$ receptor structural models do not contain a contiguous pocket linking β3H267 with the residues labeled by mTFD-MPAB and azi-Pm. Franks[17] conducted docking calculations for propofol in the β3 crystal structure that shows two separated pockets (Fig 7C) and found these consistent with o-PD photolabeling of β3 homomers [6]. Jayakar et al[12] also describe an α1β3 model based on Gloeobacter violaceus ligand gated ion channel where β3H267 forms part of a pocket adjacent to the ion channel and separated by intruding side-chains from inter-subunit residues photolabeled by azi-Pm. The accuracy of structural models *vis-a-vis* the various functional states of α1β3γ2L and other $GABA_A$ receptors remains speculative. Small helix rotations or side-chain rearrangements in the models in figures 7B and 7C could alter the shape and contiguity of the depicted pockets. Our current protection results favors a structure for α1β3γ2L receptors with "β−" anesthetic binding pockets contiguously linking the o-PD, azi-Pm, and mTFD-MPAB photolabeled residues.

Analysis of other β3H267 mutations in α1β3γ2L may provide further insights into its roles in anesthetic modulation. However, functional analysis alone may not distinguish between mutant-associated changes in anesthetic binding vs. transduction [22,29]. This is because anesthetics are highly efficacious agonists of $GABA_A$ receptors, binding almost exclusively to activated and desensitized states. In contrast, cysteine modification-protection has identified likely anesthetic contact even at residues where cysteine substitution did not significantly alter sensitivity to anesthetic [20]. This further highlights the importance of complementary methods to probe both functional and steric interactions between drug and receptor.

In summary, in cysteine modification-protection studies of α1β3H267Cγ2L $GABA_A$ receptors and four potent general anesthetics (propofol, etomidate, alphaxalone, and mTFD-MPAB), only mTFD-MPAB slowed β3H267C modification, indicating steric proximity. The β3H267C mutation also selectively enhanced direct agonism by both propofol and mTFD-MPAB. These results are consistent with a structural model locating β3H267 near the "β−" inter-subunit clefts where photolabeling indicates that both mTFD-MPAB and propofol (but not etomidate or alphaxalone) bind.

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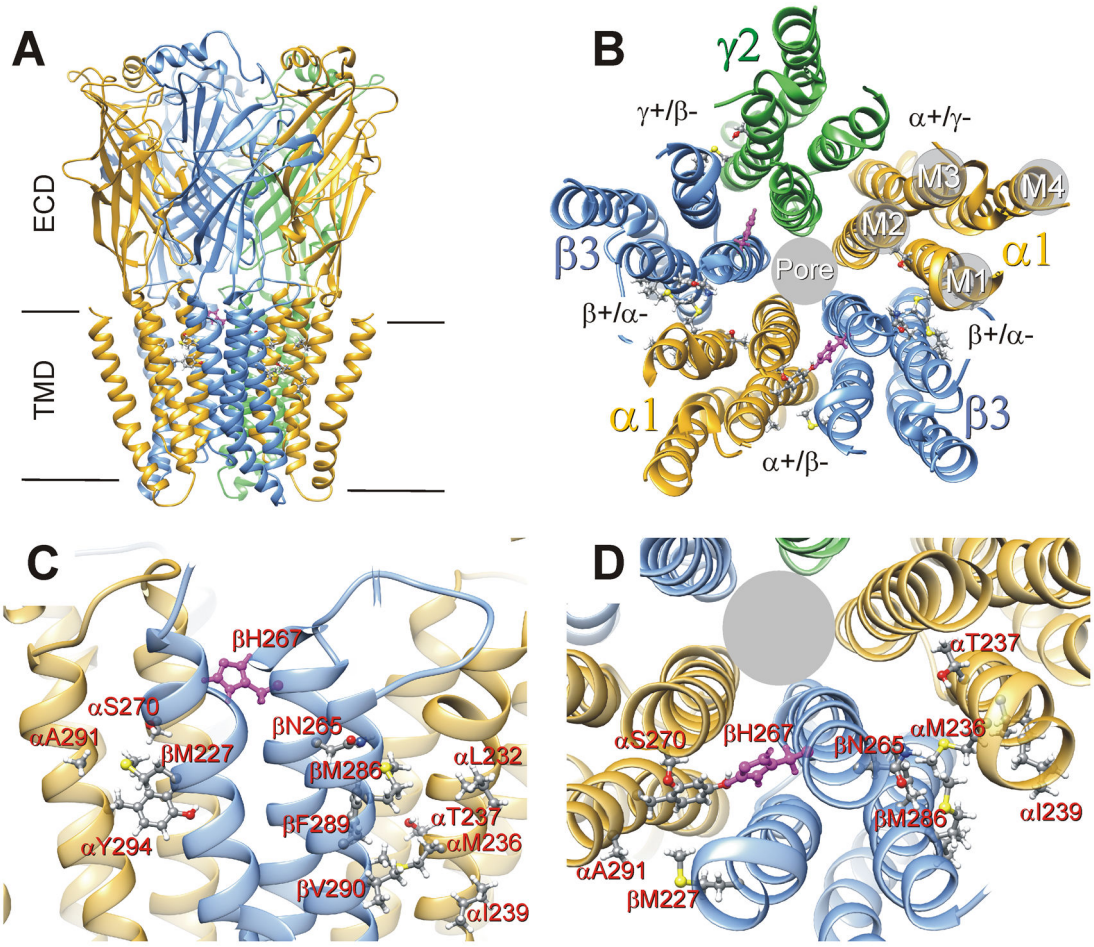

Figure 1. Anesthetic Binding Sites in a Structural Model of α1β3γ2L GABA$_A$ Receptors

A: The panel depicts a structural homology model of α1β3γ2L GABA$_A$ receptors[22], viewed from the side. Subunits are color-coded: α1 = gold, β3 = blue, and γ2 = green. The peptide chain backbones are depicted as ribbons and loops. The extracellular (ECD) and transmembrane (TMD) domains are labeled. Intracellular domains have been truncated to match those of the GluCl template. **B:** The transmembrane domain viewed from the extracellular space, depicting the established subunit arrangement, the four-helix bundles of each subunit, and the transmembrane pockets formed at subunit interfaces. Amino acid residues thought to interact with anesthetics based on either photolabeling or cysteine modification and protection (Table 1) are identified as ball-and-stick structures. The two β3H267 residues (highlighted in magenta) are located in the α+/β− and γ+/β− interfaces. **C:** A close-up view from a perspective similar to that in Panel A, identifying putative anesthetic contact residues in the α+/β− interface (on the left) and one of the β+/α− interfaces (on the right). **D:** A close-up view of the same two transmembrane interfacial pockets from the extracellular space. A subset of the putative anesthetic contact residues, including β3H267, is labeled.

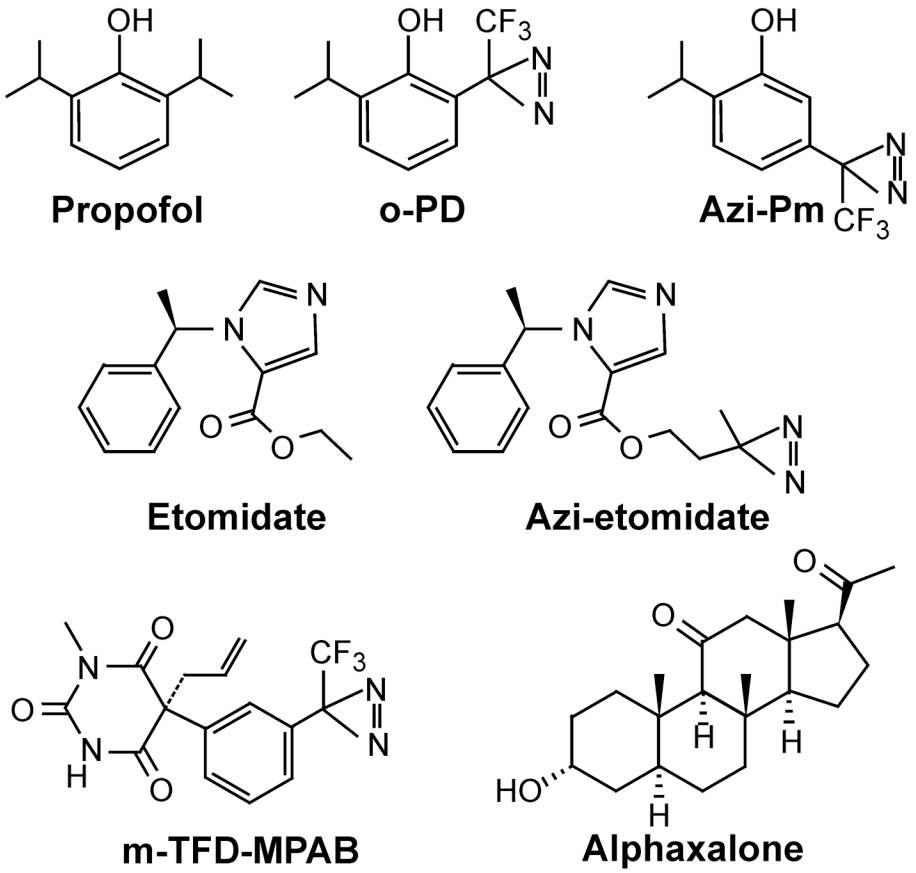

Figure 2. Potent General Anesthetics and Anesthetic Photolabels

The chemical structures of three potent anesthetics (etomidate, propofol, and alphaxalone) and four diazirine photolabels (o-PD = o-propofol diazirine; azi-Pm = m-azi-propofol; azi-etomidate, and mTFD-MPAB = *R*-5-allyl-1-methyl-5-[*m*-trifluoromethyl-diazirinylphenyl] barbituric acid) are shown.

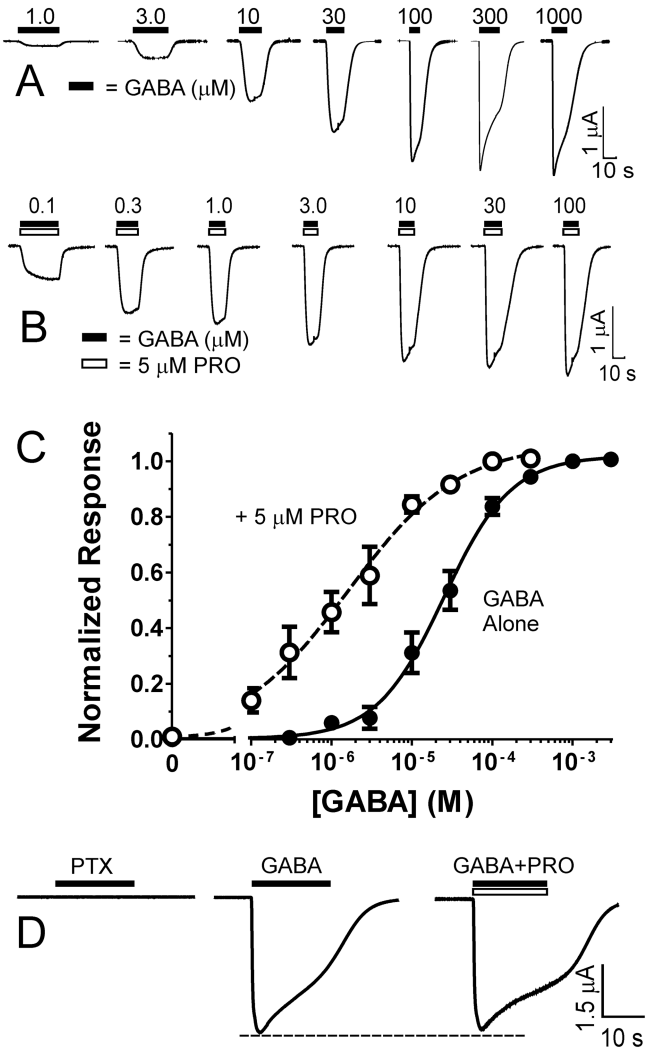

Figure 3. Functional Characterization of α1β3H267Cγ2L $GABA_A$ Receptors

A: Traces are currents measured from a single voltage-clamped oocyte expressing α1β3H267Cγ2L $GABA_A$ receptors. Bars over the traces identify GABA concentration (μM) and period of exposure. **B:** Traces are recorded from the same oocyte as panel A, activated with various GABA concentrations combined with 5 μM propofol (PRO). **C:** Combined GABA concentration-responses from 3 oocytes in the absence and presence of propofol. Normalized data was fitted with Eq. 1 (methods). Fitted GABA EC_{50} values are 25 μM with GABA alone, and 1.6 μM in the presence of 5 μM propofol. **D:** Picrotoxin (PTX) application to a voltage-clamped oocyte expressing α1β3H267Cγ2L receptors reveals an absence of spontaneous gating activity. Combining propofol (10 μM) with maximal (1 mM) GABA does not enhance peak current, indicating that GABA alone activates nearly 100% of receptors.

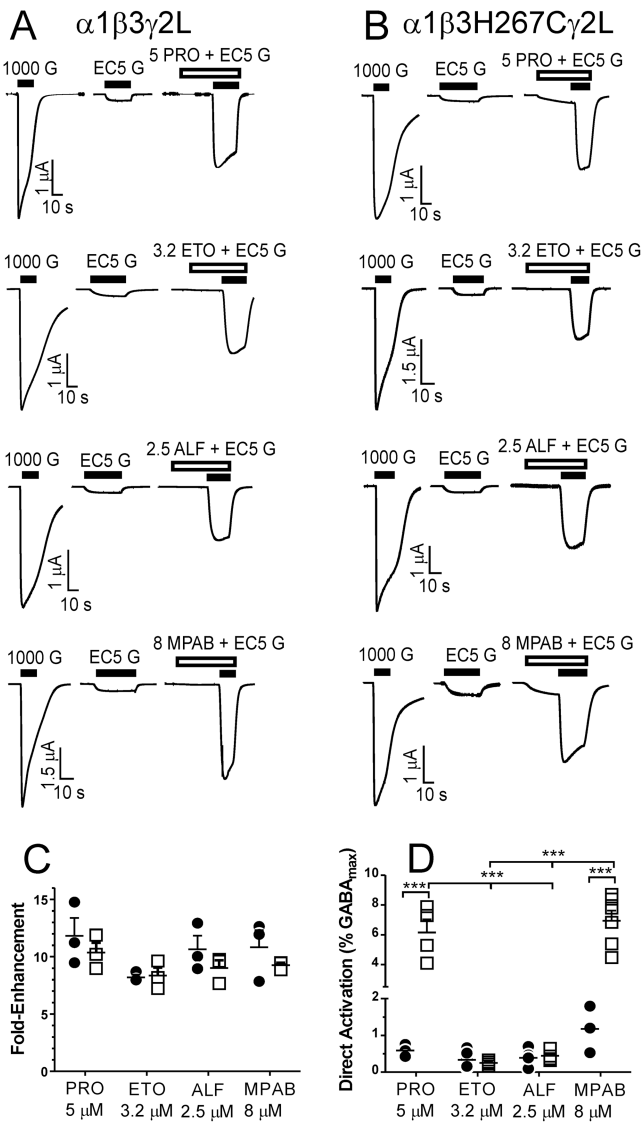

Figure 4. Anesthetic Direct Activation and Enhancement of GABA EC5 in α1β3γ2L and α1β3H267Cγ2L GABA$_A$ Receptors

A: Each set of traces is from a single oocyte expressing α1β3γ2L receptors, tested with a different anesthetic drug (PRO = propofol; ETO = etomidate; ALF = alphaxalone; MPAB = *R*-5-allyl-1-methyl-5-[*m*-trifluoromethyl-diazirinylphenyl] barbituric acid). The first trace depicts response to 1 mM GABA, the second to EC5 GABA (ranging from 3 to 6 μM), and the third shows current elicited during exposure to anesthetic (at 2 × EC50 for loss of righting reflexes in tadpoles, indicated in μM) then anesthetic plus EC5 GABA. Anesthetic concentrations are indicated in μM. **B:** The traces are from oocytes expressing α1β3H267Cγ2L receptors, studied as described for panel A. **C:** A scatter plot showing all EC5 enhancement results with α1β3γ2L (solid circles) and α1β3H267Cγ2L (open squares), using equipotent concentrations of four anesthetics. Each drug produced similar EC5 enhancement in both receptors, and the amount of enhancement was similar among the four drugs ($p > 0.05$ with two-way ANOVA). **D:** A scatter plot showing all direct activation

results with α1β3γ2L (solid circles) and α1β3H267Cγ2L (open squares). Direct activation was similar for all drugs in α1β3γ2L, but both propofol and mTFD-MPAB activated α1β3H267Cγ2L receptors much more than the other drugs and more than wild-type receptors ($p < 0.001$ for both drug and receptor types, using two-way ANOVA and Bonferroni post-tests). *** $p < 0.001$.

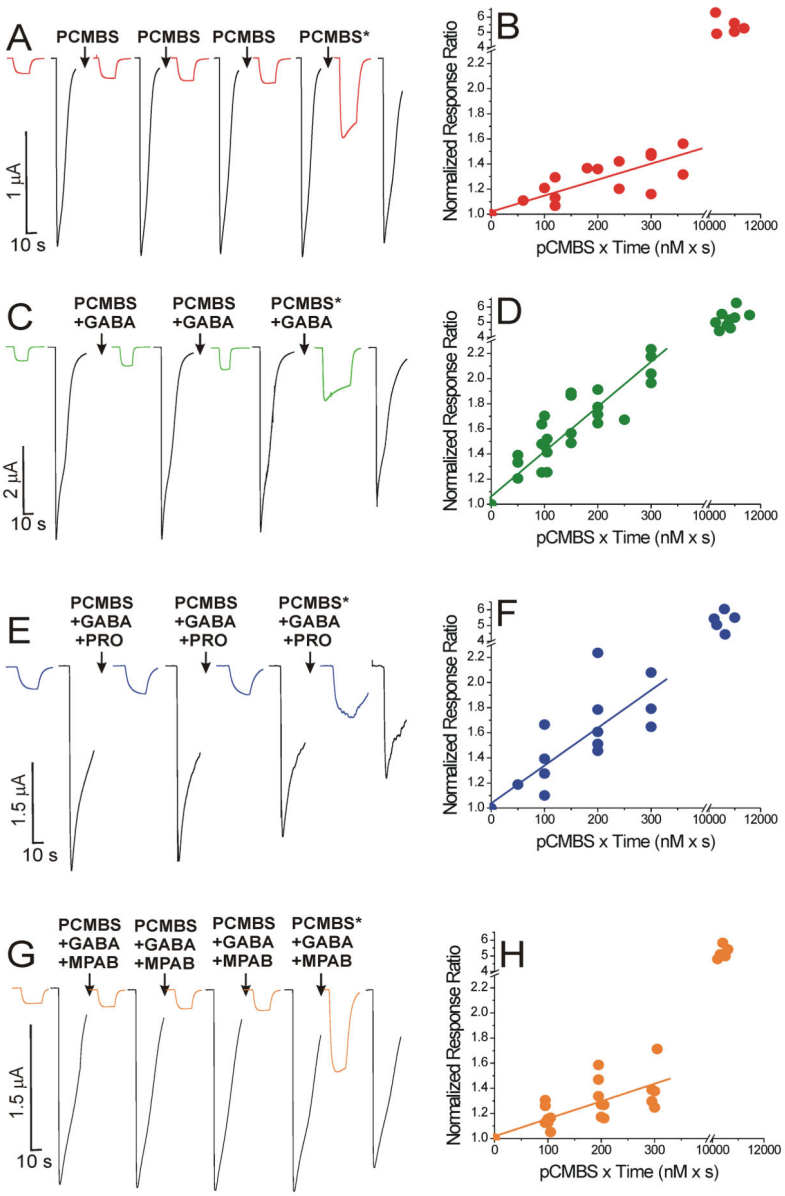

Figure 5. Modification of α1β3H267Cγ2L $GABA_A$ Receptors with pCMBS

The panels on the left show examples of voltage-clamp current traces during modification under four different conditions. Colored traces are responses to 3 µM GABA, and black traces are responses to 1 mM GABA. Arrows indicate modification exposures, which were followed by 5 min wash. The starred arrows indicate exposure to 1 µM *p*-chloromercuribenzensulfonate (pCMBS) for 10 s. The panels on the right show the corresponding initial linear rate analyses for combined normalized response $I_{3\mu M}/I_{1mM}$ ratios from all oocytes used for each condition. Points represent the ratio of $I_{3\mu M}$:I_{1mM}, normalized to the pre-modification control, and plotted against cumulative pCMBS exposure. Points in the upper right portion of the panel represent response ratios after modification with 1 µM pCMBS. **A: Modification in the absence of GABA.** Traces are recorded from one voltage-clamped oocyte expressing α1β3H267Cγ2L $GABA_A$ receptors

before and after sequential 10 s exposures to 10 nM pCMBS. **B:** Initial modification rate analysis for combined data from all oocytes modified with pCMBS alone (n = 5). The line through the first four points has a fitted slope of 1.3 ± 0.19 × 10^6 $M^{-1}s^{-1}$. Maximal normalized response ratio = 5.4 ± 0.25 (n =5; mean ± sem). **C: Modification in the presence of GABA.** Current responses from a single oocyte during sequential 10 s exposures to 10 nM pCMBS plus 1 mM GABA. **D:** Initial modification rate analysis for all oocytes modified with pCMBS plus GABA (n = 9). The fitted linear slope is 3.6 ± 0.25 × 10^6 $M^{-1}s^{-1}$. Maximal normalized response ratio = 5.2 ± 0.24 (n =8; mean ± sem). **E: Modification in the presence of GABA and propofol.** Current responses from one oocyte before and after sequential 10 s exposures to 10 nM pCMBS plus 1 mM GABA plus 10 μM propofol (PRO). **F:** Initial modification rate analysis for all oocytes modified with pCMBS plus GABA and propofol (n = 5). The fitted linear slope is 3.0 ± 0.47 × 10^6 $M^{-1}s^{-1}$. Maximal normalized response ratio = 5.3 ± 0.27 (n =5; mean ± sem). **G: Modification in the presence of GABA and mTFD-MPAB.** Current responses from one oocyte before and after sequential 10 s exposures to 10 nM pCMBS plus 1 mM GABA plus 8 μM mTFD-MPAB (MPAB = *R*-5-allyl-1-methyl-5-[*m*-trifluoromethyl-diazirinylphenyl] barbituric acid). **H:** Initial modification rate analysis for all oocytes modified with pCMBS plus GABA and MPAB (n = 7). The fitted linear slope is 1.4 ± 0.22 × 10^6 $M^{-1}s^{-1}$. Maximal normalized response ratio = 5.2 ± 0.18 (n =5; mean ± sem).

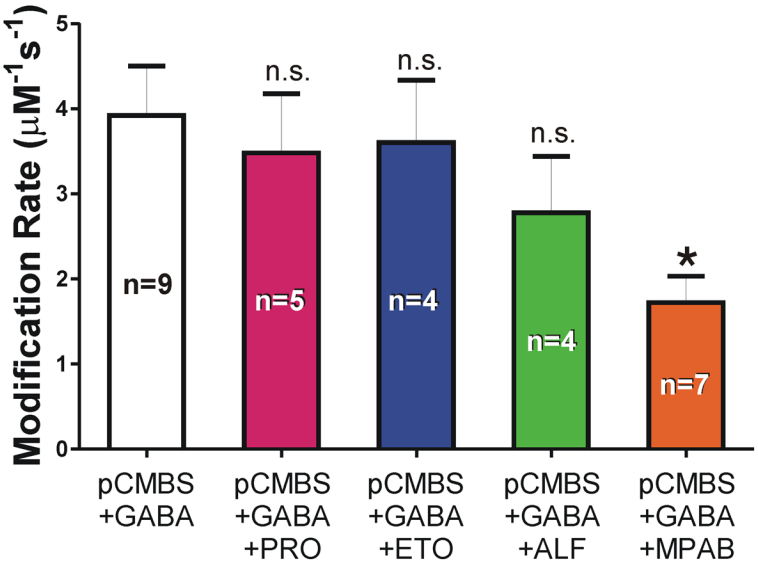

Figure 6. Anesthetic Effects on β3H267C Sulfhydryl Modification Rates

Each column represents group mean ± sem calculated from individual oocyte modification rate results. Modification conditions are labeled: 10 nM pCMBS; 1 mM GABA; PRO = 10 μM propofol; ETO = 10 μM etomidate; ALF = 10 μM alphaxalone; MPAB = 8 μM mTFD-MPAB. Results with anesthetics were compared to pCMBS plus GABA (Kruskal Wallace with Dunn's multiple comparisons), indicating that only MPAB significantly slowed modification. * p = 0.011.

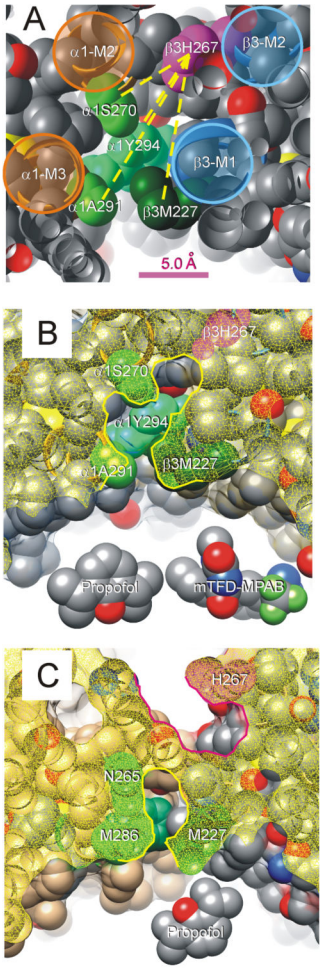

Figure 7. β3H267 and Other α+/β-Anesthetic Contact Residues Line a Contiguous Pocket

A: A portion of our α1β3γ2L structural homology model is shown with peptide backbone as ribbons and side-chains depicted as spherical shells (hydrogens are hidden). The view is from the extracellular space, off-axis, through a planar cut (atoms cut by this plane appear hollow). The peptide backbones of transmembrane helices are highlighted and labeled. The side-chain of β3H267 is colored magenta, and other side-chains known to contribute to anesthetic binding are shaded in green and labeled. Other side-chain atoms are color coded (gray = carbon, red = oxygen; blue = nitrogen; yellow = sulfur). Some side-chains (β3L223, β3Q224, α1R274, α1M286, and α1D287) were hidden in order to un-roof the cavity that contacts residues of interest. Yellow dotted lines connecting β3H267 to other side-chains represent measured distances in the model, which range from 7.0 Å (to α1S270) to 12.8 Å (to α1A291). **B:** A view of our homology model similar to that in panel A is shown. The protein surface has been added and is depicted as a translucent film. The "cut plane" is about 1 helical turn (4 Å) more intracellular than that in panel A, and the cut surface shown as yellow mesh. The highlighted border of the cut surface outlines the proposed anesthetic-binding pocket that is lined by β3H267 (magenta) and the other residues that contribute to anesthetic binding (green). Models of propofol and mTFD-MPAB are included for size comparison. **C**: A "cut" view of the crystallized β3 homomeric receptor structure[6]. The cut

surface is again shown as a yellow mesh. Note that H267 (magenta) forms part of a pocket (highlighted in red) adjacent to the ion channel. Sidechains of P228 and T266 separate the pocket containing H267 from another (highlighted in yellow) that includes other anesthetic photolabeled residues (green) and part of the lipid-protein interface.

Table 1

Anesthetic Contact Residues in $GABA_A$ receptors

Residue	Receptor Type	Interfacial Sites	Photolabels	Substituted Cysteine Modification-Protection
α1L232	α1β3γ2L	β+/α−	—	ETO[20]
α1M236	αβγ, α1β3	β+/α−	Azi-ETO[14]	ETO, PRO[20,22]
	α1β3	“	TDBzl-ETO[32]	ND
	α1β3	“	Azi-Pm[12]	ND
α1T237	α1β3γ2L	β+/α−	—	ETO[20]
α1I239	α1β3	β+/α−	Azi-Pm[12]	— [20], *a*
α1S291	α1β3γ2L	α+/β−, α+/γ− *b*	mTFD-MPAB[15]	ND
α1Y294	α1β3γ2L	α+/β−, α+/γ− *b*	mTFD-MPAB[15]	ND
β3M227	α1β3	α+/β−, β+/β−	Azi-Pm[12]	ND
	α1β3γ2L	α+/β−, γ+/β−	mTFD-MPAB[15]	
β3N265	α1β3γ2L	β+/α−	—	ETO, PRO[22], *c*
β3H267	**β3, α1β3**	**β+/β−, α+/β−**	**o-PD**[13]	*d*
β3M286	αβγ, α1β3	β+/α−, β+/β−	Azi-ETO[14]	ETO, PRO[19,21]
	α1β3	“	TDBzl-ETO[32]	ND
	α1β3	“	Azi-Pm[12]	ND
	α1β3	β+/β− *e*	mTFD-MPAB[12]	ND
β3F289	α1β3	β+/β− *e*	mTFD-MPAB[12]	ND
β3V290	α1β3	β+/α−, β+/β−	TDBzl-ETO[32]	ND
γ2S301	α1β3γ2L	γ+/β−	mTFD-MPAB[15]	ND

Azi-ETO = azi-etomidate; TDBzl-ETO = o-trifluromethyldiazirinylphenyl-etomidate; Azi-Pm = *m*azi-propofol; o-PD = *o*-propofol diazirine; mTFD-MPAB = *R*-5-allyl-1-methyl-5-(*m*-trifluoromethyl-diazirinylphenyl) barbituric acid; — Indicates negative modification or protection result; ND indicates no published data.

a *p*-Chloromercuribenzenesulfate application to α1I239C did not alter function.

b To date, there is no evidence of anesthetic contact with γ-M1 helix residues, so anesthetic binding in the α+/γ− interface remains speculative.

c A binding role for βN265 was indirectly demonstrated using α1M236C protection.

d Current study.

e mTFD-MPAB did not photolabel βM286 or β3F289 in α1β3γ2L, but did in α1β3. Thus, incorporation into these residues was likely at the β+/β− interface.