Title: Substituted Cysteine Modification and Protection with n-Alkyl-MTS Reagents Quantifies Steric Changes Induced by a Mutation in Anesthetic Binding Sites on GABA Type A Receptors*

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Abstract

Multiple approaches, including cryo-EM, indicate that the anesthetics etomidate and propofol modulate α1β2/3γ2 GABA_A receptors by binding in overlapping transmembrane inter-subunit sites near βM286 and αL232 sidechains. High precision approaches in functional receptors are needed for comparisons with cryo-EM. We previously used substituted cysteine modification and protection (SCAMP) with n-alkyl-methanethiosulfonate (MTS) reagents and electrophysiology in α1β3M286Cy2L receptors, to estimate the distance from etomidate to β3M286 with precision near 1.3 Å. Here, we address three more aims using this approach: 1) SCAMP with etomidate was tested in α1L232Cβ3γ2L receptors; 2) Studies in α1L232Wβ3M286Cy2L receptors assessed whether α1L232W displaces etomidate relative to β3M286C; and 3) Results with propofol were compared to those with etomidate. Voltage-clamp electrophysiology in Xenopus oocytes was used to assess persistent functional changes after exposing cysteine substituted receptors to methyl-MTS through n-decyl-MTS. Overlap of modified cysteine sidechains with bound anesthetic was inferred when anesthetic co-application with alkyl-MTS reagent blocked development of persistent effects. In α1L232Cβ3γ2L receptors, only pentyl-MTS and hexyl-MTS induced persistent effects that were unaltered by etomidate co-application, precluding a direct estimate of intermolecular distance. In α1L232Wβ3M286Cy2L receptors, sidechain overlap with bound etomidate was inferred for modifications with ethyl-MTS through n-pentyl-MTS, with unambiguous cut-on and cut-off. Comparison to results in α1β3M286Cy2L reveals that α1L232W, which increases maximal sidechain length by 2.1 Å, displaces etomidate closer to β3M286C by about 1.3 Å. Propofol results largely mirrored those with etomidate. These findings indicate that both etomidate and propofol bind within 1 Å of α1L232, consistent with cryo-EM structures.
Significance Statement

We combined electrophysiology, cysteine substitutions, and n-alkyl-methanethiosulfonate modifiers in functional GABA<sub>A</sub> receptors to enable precise estimates of the distance between β3M286C sidechains and anesthetics (etomidate and propofol) bound in transmembrane β+/α-inter-subunit pockets. Comparing results in α1β3M286Cγ2L and α1L232Wβ3M286Cγ2L receptors reveals that α1L232W mutations displace both anesthetics toward β3M286C, indicating that these anesthetics bind within 1 Å of the α1L232 sidechain in functional receptors, consistent with cryo-EM structures derived under non-physiologic conditions.
Introduction

GABA<sub>\text{A}</sub> receptors are pentameric ligand-gated chloride ion channels that inhibit neuronal activity in the central nervous system when activated (Sigel and Steinmann, 2012). A variety of intravenous sedative-hypnotics, including etomidate and propofol, act as allosteric agonists and positive modulators of GABA<sub>\text{A}</sub> receptors (Hemmings et al., 2019), and transgenic animal studies indicate that β3-containing receptors mediate important behavioral effects of these drugs (Jurd et al., 2003; Yang et al., 2019). Synaptic α1β2/3γ2L GABA<sub>\text{A}</sub> receptors, which contain 2α, 2β and 1γ subunits arranged β-α-β-α-γ counterclockwise from an extracellular perspective, have been the focus of most studies of anesthetics. In these receptors, overlapping binding sites for etomidate and propofol have been located in transmembrane interfacial pockets between β and α subunits (β+/α- interfaces) (Forman and Miller, 2016). Convergent evidence for this model has come from mutant function analyses (Krasowski et al., 2001; Nourmahnad et al., 2016; Stewart et al., 2008), photolabeling (Jayakar et al., 2014; Li et al., 2006), substituted cysteine modification and protection (SCAMP) (Nourmahnad et al., 2016; Stewart et al., 2013b) and cryogenic electron-microscopy (cryo-EM) (Kim et al., 2020). Each of these approaches has limitations, making correlations between data from different experimental approaches essential for mechanistic understanding and drug design. Notably, spatial resolution varies among these approaches; photolabeling and mutational analyses limit inferences to the scale of amino acid residues, while cryo-EM images of α1β2γ2L receptors bound to anesthetics (Kim et al., 2020) provide resolution near 3.5 Å. SCAMP, which senses steric interactions between a protective ligand (e.g. anesthetic drug) and cysteine-specific modifiers that alter receptor function under physiological conditions, provides spatial resolution dependent on the modifier size. Using SCAMP with a series of n-alkyl-methanethiosulfonate (alkyl-MTS) reagents and electrophysiology, we investigated the distance between etomidate and the β3M286C sulfhydryl group in α1β3M286Cy2L receptors (Fantasia et al., 2021). This approach provides spatial...
resolution approaching 1.3 angstroms, the alkyl chain-length difference produced by addition of a single methyl group at a dihedral angle of 109.5°.

The βM286C mutation proved advantageous for these studies because its function has been previously characterized (Stewart et al., 2013a). It is readily modified by alkyl-MTS reagents resulting in robust functional changes. Modification by reagents from propyl-MTS to hexyl-MTS was inhibited by bound etomidate (Fantasia et al., 2021). Propofol also protects βM286C from modification by the large rigid sulphydryl modifier para-chloromercuribenzenesulfonate (pCMBS) (Bali and Akabas, 2004; Ziemba et al., 2018). In cryo-EM structures, α1L232 residues are positioned opposite β3M286 on α1-M1 transmembrane helices abutting the β+/α- clefts where propofol and etomidate bind. Receptors with bulky α1L232W mutations impair modulation by both etomidate and propofol, possibly by sterically affecting drug binding (Nourmahnad et al., 2016). However, activated receptors containing βM286C mutations form disulfide cross-links with αQ229C and αY225C, but not with αL232C (Bali et al., 2009; Borghese et al., 2014). Modification of α1L232C by pCMBS is blocked by etomidate, but not propofol (Nourmahnad et al., 2016), suggesting that these drugs bind similar distances from βM286 but different distances from α1L232.

In the present study, we applied SCAMP and alkyl-MTS reagents to: 1) test whether varying size modifications at α1L232C can assess distances between this residue and bound etomidate; 2) test whether the α1L232W mutation affects the size of alkyl-MTS reagents that modify β3M286C and/or displaces etomidate relative to β3M286C; and 3) compare propofol effects to those of etomidate on alkyl-MTS modification of β3M286C in α1β3M286Cy2L and α1L232Wβ3M286Cy2L receptors. Two-electrode voltage clamp electrophysiology in Xenopus oocytes heterologously expressing GABA<sub>A</sub> receptors was used to assess the functional consequences of alkyl-MTS modification in the absence of anesthetics and protection from modification in the presence of etomidate or propofol.
Materials & Methods

Animals: Female *Xenopus laevis* were housed and maintained in a veterinarian-supervised facility with approval from the Massachusetts General Hospital Institutional Animal Care and Use Committee (protocol #2005N000051) and in line with ARRIVE guidelines. Frogs were anesthetized by immersion in 0.2% tricaine for oocyte harvest procedures via mini-laparotomy, in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Materials: N-Alkyl-methanethiosulfonate reagents (methyl-MTS, ethyl-MTS, n-propyl-MTS, n-butyl-MTS, n-pentyl-MTS, n-hexyl-MTS, n-octyl-MTS and n-decyl-MTS) and p-chlorobenzenesulfonate (pCMBS) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). N-alkyl-MTS reagents and pCMBS were prepared as 1M stocks in DMSO and stored at -20 °C. Stock solutions were diluted in electrophysiology buffer on the day of experiments. Sulfhydryl reactivity of reagents was assayed as described by Karlin and Akabas (1998) when received and every 2 months afterward. Sulfhydryl-modifying reagents were replaced when reactivity of stock solutions dropped below 80% of initial value. R-etomidate was a gift from Prof. Douglas E. Raines (Department of Anesthesia Critical Care & Pain Medicine, Massachusetts General Hospital Boston, MA) and synthesized by Bachem America (Torrance, CA). Propofol (2,6-diisopropyl phenol) was purchased from Sigma-Aldrich (St. Louis, MO). Etomidate and propofol were prepared as 100mM stocks in DMSO and stored at -20 °C. Anesthetic stock solutions were diluted in electrophysiology buffer on the day of experiments. γ-Aminobutyric acid (GABA), buffers, salts, and antibiotics were all purchased from Sigma-Aldrich.

Molecular Biology: Complementary DNAs encoding human GABA<sub>A</sub> receptor subunits α1, β3, and γ2L in pCDNA3.1 expression vectors were used. Construction of plasmids encoding β3M286C, α1L232C, and α1L232W mutations were previously described (Nourmahnad et al., 2016; Stewart et al., 2013a). Capped messenger RNAs were synthesized *in vitro* on linearized
DNA templates using mRNA templates using mMessage mMachine kits (Thermo Fisher, Waltham, MA), purified, and stored in nuclease-free water at -80°C.

**Oocyte Expression of GABA<sub>A</sub> Receptors:** Harvested oocytes were defolliculated by enzymatic digestion (Forman and Stewart, 2012) and later injected with messenger RNA mixtures encoding wild-type or mutant GABA<sub>A</sub> receptor subunits in 1α:1β:5γ ratios (final concentration 1ng/nl). Injected oocytes were incubated at 18°C in ND-96 (96mM NaCl, 3mM KCl, 1.8mM MgCl₂, 5 mM Hepes, pH 7.4) supplemented with 0.05 mg/ml gentamicin, 0.1 mg/ml ampicillin, and 0.025 mg/ml ciprofloxacin.

**Two Electrode Voltage-Clamp Electrophysiology.** Electrophysiology experiments were performed at room temperature (21-23°C), 16-48 hours after messenger RNA injection. Oocytes were positioned in a custom-built low-volume (0.2 ml) flow chamber and impaled with microelectrodes containing 3M KCl (resistance 0.5 to 2 MΩ) and voltage-clamped at -50 mV (model OC-725C; Warner Instruments, Hamden, CT). During cysteine modification and electrophysiological recordings of GABA<sub>A</sub> receptor-mediated currents, oocytes were superfused with various solutions in ND-96, delivered at 4ml/min from glass syringe reservoirs via computer-controlled valves (VC-8; Warner Instruments) and a micromanifold (ALA Scientific Instruments). Transmembrane currents in voltage-clamped oocytes were digitized at 200 Hz on a computer running ClampEx version 8.0 software (Molecular Devices, San Jose, CA). Data were digitally filtered with a 10-Hz low-pass Bessel function and analyzed off-line using Clampfit8 software (Molecular Devices).

**GABA-activated Responses and Anesthetic Modulation:** Each GABA<sub>A</sub> receptor type studied was characterized to establish its relative activation by varying GABA concentrations. Voltage-clamped oocytes were exposed to GABA solutions (concentration range 0.1 µM to 10 mM) for 30s, followed by a 5-minute wash in ND-96. Normalized GABA-dependent activation was fit using non-linear least squares to a logistic dose-response function,
Y=1/(1+10^((LogEC50-X)*nH)), where Y is the fraction of maximal activation, X is log[GABA], EC_{50} is the half-maximal effect GABA concentration, and nH is the Hill slope. The GABA concentration eliciting 3% of maximum (EC3) was established for each GABA\textsuperscript{A} receptor type studied. In separate sets of oocytes, anesthetic-dependent modulation of EC3 current responses was assessed. Voltage-clamped oocytes were exposed to etomidate or propofol for 30s before exposure to anesthetic + EC3 GABA for 30-60s, followed by 5-min wash in ND-96. Normalization currents elicited with maximal (3 to 10 mM) GABA were recorded every other trace. Peak currents for each tested GABA concentration were normalized to the average of the preceding and following maximum GABA responses. 5 oocytes were used for each condition studied.

**Spontaneous Receptor Activity and GABA Intrinsic Efficacy:** Consistent with previous studies of receptors containing the mutations we used (Nourmahnad et al., 2016; Stewart et al., 2013a), none of the mutant receptors demonstrated high leak currents suggestive of spontaneous receptor activity. This was confirmed by testing whether 2 mM picrotoxin produced outward currents in oocytes expressing mutant receptors. These tests were consistently negative (n = 5 oocytes each). The maximal intrinsic efficacy of GABA for each receptor type was assessed by comparing currents elicited with 10 mM GABA to those elicited with 10 mM GABA plus 3 to 10 μM alphaxalone, a positive modulator that is unaffected by the mutations used in this study (n = 5 each).

**Cysteine Modification with Alkyl-MTS reagents:** Cysteine modification of mutant receptors was performed by exposing oocytes to solutions containing maximally-activating GABA (3 or 10 mM) plus alkyl-MTS reagents for time periods resulting in 9 mM x s exposures. These are maximally modifying conditions that were first established for α1β3M286Cγ2L receptors (Fantasia et al., 2021): n-decyl-MTS and n-octyl-MTS: 50 μM for 180 seconds, n-Hexyl-MTS and n-Pentyl-MTS: 100 μM for 90 seconds, methyl-MTS, ethyl-MTS, n-propyl-MTS and n-butyl-
MTS: 300 μM for 30 seconds. Longer exposures to the most efficacious modulators were tested in α1L232Cβ3γ2L and α1L232Wβ3M286Cy2L receptors but did not further increase the functional effects.

**Modification Effects on GABA Sensitivity-- Low:High Modification Ratios:** Functional effects of cysteine modification were assessed electrophysiologically through changes in receptor sensitivities to both GABA and anesthetics, as previously described (Fantasia et al., 2021). Briefly, voltage-clamped oocyte currents were first elicited with high GABA (3 or 10 mM) for 15 seconds (sweep 1), followed by a five-minute wash in ND96. A second current sweep (sweep 2) was then activated with low GABA (EC3) for 30 seconds, followed by another 5-minute ND-96 wash. Alkyl-MTS reagents were co-applied with GABA only to oocytes exhibiting two consecutive low:high GABA response ratios between 0.01 and 0.05 and differing by less than 20%, indicating stable baseline responses. After a 10-minute wash in ND-96, post-exposure current responses to high GABA, then low GABA, with intervening ND-96 washes, were again recorded in duplicate. Control studies for each receptor type used exposure to high GABA alone (with no alkyl-MTS reagent).

Low:high GABA response ratios before and after exposure to n-alkyl-MTS reagents were calculated for each set of 2 sweeps as the ratio of the EC3 GABA current response (peak of sweep 2) to the high GABA response (peak of sweep 1). Low:high modification ratios for each oocyte were calculated as the average post-exposure low:high GABA response ratio to the average pre-exposure low:high GABA response ratio. Each modification condition was studied in 5 oocytes and accompanied by controls in 5 oocytes.

**Modification Effects on Anesthetic Sensitivity-- Modulation Modification Ratios:** To assess anesthetic modulation, the second current sweep (sweep 2) was initially activated with EC3 GABA for 30 seconds, then with EC3 GABA + anesthetic for 45-60 seconds, followed by another 5-minute ND-96 wash. Anesthetic modulation ratios were calculated for each set of
current sweeps as the ratio of current elicited with EC3 GABA + anesthetic (second peak in sweep 2) to the EC3 GABA response (first peak in sweep 2). Anesthetic modulation modification ratios for each oocyte were calculated as the average post-exposure modulation ratio to the average pre-exposure modulation ratio. Anesthetic concentrations used in sweep 2 were chosen for each receptor type to produce pre-modification modulation ratios near 5.0: for α1L232Cβ3γ2L receptors, 10 µM etomidate; for α1β3M286Cy2L receptors, 100 µM etomidate and 30 µM propofol; for α1L232Wβ3M286Cy2L receptors, 300 µM etomidate and 30 µM propofol.

**Anesthetic Protection from Alkyl-MTS Modification:** For alkyl-MTS reagents that produced low:high GABA modification ratios significantly different from control (i.e. p < 0.05; see Statistical analysis below), we tested whether the presence of anesthetics during n-alkyl-MTS exposures blocked the development of modification effects. After assessing pre-modification low:high GABA response ratios and 5-minute wash, oocytes (n = 5 per alkyl-MTS reagent) were exposed to anesthetic (300 µM etomidate or 50 to 150 µM propofol) for 30 s, then to anesthetic + maximal GABA + n-alkyl-MTS reagents (9 mM x s). After additional ND-96 washes of 10 min, post-modification low:high GABA response ratios were re-assessed in duplicate. Because both etomidate and propofol enhanced the 3 mM GABA current responses in the mutant receptors, control modifications for protection experiments were also performed using exposures to GABA + 3 µM alphaxalone + alkyl-MTS, conditions that maximized receptor activation (Stewart et al., 2013a) using a modulator that does not bind near β3M286 or α1L232. Five oocytes were used for each protection condition along with 5 controls. Addition of alphaxalone did not alter control modification (GABA + 9 mM x s alkyl-MTS exposure) effects in any of the receptors we studied.

**Molecular modeling and measurements:** Molecular models of modified cysteine sidechains were constructed using a web-based Molecular Editor

were visualized, energy-minimized, and measured using JSMol, an open-source HTML5 viewer for chemical structures (http://wiki.jmol.org/index.php/JSmol).

Cryogenic electron microscopy structures of α1β2γ2L GABA_α receptors with bound etomidate (PDB 6X3V) or propofol (PDB 6X3T), both from Kim et al (2020), were visualized and analyzed using the UCSF Chimera package (v 1.13) (Pettersen et al., 2004), developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).

**Statistical Analysis:** Non-linear least-squares fits and statistical analyses were performed in GraphPad Prism v 9.3.1 (GraphPad Software, San Diego, CA). Results are reported and displayed as mean ± standard deviation or mean (95% confidence interval). Single variable ANOVA analysis and Dunnett’s multiple comparisons tests were used to compare GABA low:high modification ratios and anesthetic modulation modification ratios for each of the n-alkyl-MTS reagents (n = 5 per reagent) with controls exposed to GABA alone (n = 5 per reagent). Comparisons between two experimental groups were based on unpaired Student’s t-tests. Statistically significant results were inferred with p<0.05. We inferred that anesthetics sterically obstructed sulfhydryl modification by an n-alkyl-MTS reagent if anesthetic presence during exposure reduced the low:high GABA response modification ratio and met the statistical threshold.
Results

Exposure of α1L232Cβ3γ2L receptors to alkyl-MTS reagents produces small increases in GABA sensitivity that are not blocked by etomidate.

We tested whether n-alkyl-MTS modification at α1L232C could provide an estimate of the distance between this residue and the etomidate site, following the approach used at β3M286C (Fantasia et al., 2021). The properties of α1L232Cβ3γ2L receptors were consistent with those previously described (Nourmahnad et al., 2016; Stewart et al., 2013b). These receptors displayed a GABA EC$_{50}$ of 61 µM (95% CI 37 to 95 µM; n = 3 oocytes), no detectable spontaneous activation, and open probability at 3 mM GABA near 100% (6% average enhancement at 3 mM GABA with addition of alphaxalone). Etomidate directly activated α1L232Cβ3γ2L receptors with an EC$_{50}$ of 32 µM (95% CI = 13 to 86 µM; n = 3 oocytes). Exposure to 3 mM GABA + pCMBS (100 µM x 30 s = 3 mM x s) increased low:high GABA response ratios about 7-fold (6.7 ± 0.49; mean ± sd; n = 6). Addition of etomidate (100 to 300 µM) inhibited the low:high GABA response ratio following pCMBS exposure by up to 65% (n = 5; p < 0.0001; F = 13.7; by ANOVA with Dunnett’s test).

FIGURE 1 HERE

Of eight alkyl-MTS reagents tested at 9 mM x s, only two induced increased sensitivity to GABA sufficiently to meet statistical significance criteria (p < 0.05 by ANOVA with Dunnett’s tests); pentyl-MTS and hexyl-MTS (Fig 1A and B). Control exposures to GABA without alkyl-MTS reagent resulted in a low:high modification ratio = 1.3 ± 0.15 (mean ± sd; n = 40; Fig 1B). Modification effects following GABA+ hexyl-MTS exposures monotonically increased with exposures to higher concentrations, achieving a maximum at 9 mM x s (Fig 1C). The maximal modification ratio for pentyl-MTS was 1.9 ± 0.26 (mean ± sd; n = 5; p < 0.0001 vs. control by ANOVA with Dunnett’s test) and for hexyl-MTS was 2.4 ± 0.40 (mean ± sd; n = 5; p < 0.0001 vs. control by ANOVA with Dunnett’s test). However, addition of etomidate at 300 µM to hexyl-MTS...
did not inhibit the effects induced by exposure to GABA + hexyl-MTS alone (Fig 1D). These results show that some alkyl-MTS reagents modify α1L232C and alter GABA sensitivity, but these modified sidechains do not impinge on the etomidate site, precluding this approach for estimating distances from α1L232C to anesthetic.

**Incorporation of α1L232W mutations reduces both cut-on and cut-off sizes for alkyl-MTS modification at β3M286C and etomidate protection.**

We next tested whether α1L232W mutations altered the size and/or position of the etomidate site by applying n-alkyl-MTS modifiers to α1L232Wβ3M286Cγ2L receptors (Fig 2) for comparison with our previous results in α1β3M286Cγ2L receptors (Fantasia et al., 2021).

GABA activation of oocytes expressing α1L232Wβ3M286Cγ2L receptors was characterized by EC50 = 107 µM (95% CI = 90 to 129 µM). Alphaxalone increased maximal GABA responses by 1.9 ± 0.08-fold (n = 5), indicating intrinsic GABA efficacy of 0.53 ± 0.023. Both etomidate and propofol modulated GABA EC3 responses and, at concentrations above 100 µM, directly agonized α1L232Wβ3M286Cγ2L receptors with low efficacies relative to GABA.

**FIGURE 2 HERE**

To study alkyl-MTS modification, we used a two-sweep protocol that assessed both low:high GABA response ratios and etomidate modulation ratios before and after exposure to high GABA plus alkyl-MTS reagents. Figure 2A shows an example of sweep pairs from an oocyte exposed to GABA + 9 mM x s hexyl-MTS, which produced only small changes in both the GABA response ratio and the etomidate modulation ratio. In contrast, exposure to pentyl-MTS (Fig 2B) produced large persistent increases in the low:high GABA response ratio and persistent decreases in the etomidate modulation ratio. In protection studies, addition of etomidate (300 µM) during pentyl-MTS modification reduced the resulting low:high GABA modification ratio (Fig 2C). Fig 2D summarizes GABA low:high modification ratios in α1L232Wβ3M286Cγ2L receptors exposed to eight different alkyl-MTS reagents ranging from methyl-MTS to decyl-MTS (n = 5
Persistent and statistically significant (p < 0.05 by ANOVA and Dunnett’s tests) increased low:high GABA modification ratios were observed following exposures to ethyl-MTS, n-propyl-MTS, n-butyl-MTS, and n-pentyl-MTS, but not following exposures to methyl-MTS, n-hexyl-MTS, n-octyl-MTS, or n-decyl-MTS (n-heptyl-MTS and n-nonyl-MTS were not available). These results indicate a cut-on between methyl-MTS and ethyl-MTS, one methyl-group smaller than prior results for α1β3M286Cy2L receptors that showed a cut-on between ethyl-MTS and propyl-MTS (Fantasia et al., 2021). Additionally, these results indicate a cut-off in modification effects beyond pentyl-MTS in α1L232Wβ3M286Cy2L receptors, a shift of at least one methyl group shorter relative to prior results in α1β3M286Cy2L receptors (Fantasia et al., 2021).

Analysis of persistent effects on etomidate enhancement following n-alkyl-MTS modification (Fig 2E) showed the same alkyl chain size dependence seen in low:high GABA modification ratios, but with inverse effects (i.e. etomidate modulation fell as GABA sensitivity increased). Etomidate enhancement modulation ratios also showed a cut-on at ethyl-MTS and a cut-off beyond pentyl-MTS. Moreover, the persistent changes in α1L232Wβ3M286Cy2L receptor function produced by the four n-alkyl-MTS modifiers were all reduced by co-application with 300 μM etomidate (Fig 2F; all p < 0.005 by Student’s t-tests). Thus, SCAMP in α1L232Wβ3M286Cy2L receptors indicates steric overlap between etomidate and modified β3M286C sidechains with both a cut-on between methyl and ethyl-MTS and a cut-off between pentyl- and hexyl-MTS. These results differ from those in α1β3M286Cy2L receptors (Fantasia et al., 2021), implying steric displacement of anesthetics by the αL232W mutation.

Propofol interactions with α1β3M286Cy2L and α1L232Wβ3M286Cy2L receptors mirror those of etomidate.

There is abundant evidence that propofol binds in the same β+/α- transmembrane interfacial pockets where etomidate acts. We studied propofol modulation following n-alkyl-MTS modification and propofol protection from n-alkyl-MTS modification in both α1β3M286Cy2L and
α1L232Wβ3M286Cγ2L receptors, for comparison with etomidate results.

In α1β3M286Cγ2L receptors, exposure to pentyl-MTS (9 mM x s) resulted in persistent low:high GABA modification ratios (7 ± 1.2; n = 5) comparable to those observed with similar exposures to butyl-MTS (9 ± 2.4; n = 5) and hexyl-MTS (5.0 ± 0.51; n = 5). Figure 3A summarizes propofol enhancement modification ratios following exposure to seven n-alkyl-MTS reagents (n = 5 each). Exposing GABA-activated α1β3M286Cγ2L receptors to methyl-MTS and ethyl-MTS produced negligible changes in propofol enhancement relative to control exposures to GABA alone, while propyl-MTS and larger modifiers up to n-octyl-MTS all induced large persistent reductions in propofol enhancement.

FIGURE 3 HERE

Fig 3B summarizes propofol protection results based on low:high GABA modification ratios after exposure to the five alkyl-MTS reagents that induced persistent functional effects in α1β3M286Cγ2L receptors. Modification in the presence of 100 µM propofol inhibited the effects of 9 mM x s exposures to propyl-MTS, butyl-MTS, pentyl-MTS and hexyl-MTS (all with p < 0.05 by Student’s t-tests), but not octyl-MTS (p = 0.36), mirroring our prior etomidate protection results (Fantasia et al., 2021).

One difference between propofol and etomidate interactions with α1β3M286Cγ2L was observed. While etomidate modulation modification ratios increased by about 20% (n = 5; p = 0.004 by ANOVA with Dunnett’s test) after ethyl-MTS exposure (Fantasia et al., 2021), propofol modulation modification ratios after ethyl-MTS exposure (Fig 3A) did not differ from control values (n = 5; p = 0.833).

In α1L232Wβ3M286Cγ2L receptors, propofol enhancement was persistently reduced following exposures to ethyl-MTS, n-propyl-MTS, n-butyl-MTS, and n-pentyl-MTS (Fig 4A). Exposures to methyl-MTS, n-hexyl-MTS, and n-octyl-MTS did not alter propofol enhancement relative to control exposures to GABA alone. These results show the same pattern observed for etomidate
enhancement following exposures to different size alkyl-MTS reagents in α1L232Wβ3M286Cy2L receptors (Fig 2E). A cut-on is evident at ethyl-MTS and a cut-off is evident beyond pentyl-MTS.

FIGURE 4 HERE

When present during alkyl-MTS exposures, propofol, like etomidate (Fig 2F), reduced the effects induced by exposures to ethyl-MTS, propyl-MTS, butyl-MTS, and pentyl-MTS (Fig 4B).

In summary, SCAMP studies using propofol and alkyl-MTS reagents in both α1β3M286Cy2L and α1L232Wβ3M286Cy2L receptors produced similar results to those using etomidate, indicating that both anesthetics have similar steric relationships to both β3M286 and α1L232 within the 1.3 Å resolution of our methods.
Discussion

We used variable-length n-alkyl-MTS reagents to covalently modify engineered cysteines located near the transmembrane β+/α- anesthetic pockets in α1β3γ2L GABA_α receptors, where both etomidate and propofol bind. We inferred that covalently modified cysteine sidechains overlapped with bound anesthetics if exposure to a modifier induced persistently enhanced GABA sensitivity and if co-applied anesthetics inhibited modification effects.

Our major findings were 1) that alkyl-MTS reagents in α1L232Cβ3γ2L receptors could not be used to directly estimate the distance to bound etomidate; 2) that incorporation of α1L232W mutations into α1β3M286Cγ2 receptors altered both cut-on and cut-off alkyl-MTS sizes for interactions with bound etomidate and 3) results for propofol interactions with alkyl-MTS modifications in both α1β3M286Cγ2L and α1L232Wβ3M286Cγ2L receptors were very similar to those for etomidate.

In α1L232Cβ3γ2L receptors, only pentyl-MTS and hexyl-MTS exposures induced statistically significant but modest functional effects (low:high GABA modification ratios near 2), and these were not blocked by etomidate. In contrast, α1L232Cβ3γ2L exposure to pCMBS produced larger persistent effects that were blocked by co-applied etomidate, in agreement with Nourmahnad et al (2016).

Hexyl-MTS modification increases cysteine sidechain length by up to 9.4 Å, more than the 9.1 Å length increase with pCMBS. Thus, for the α1L232C mutation, modifier length alone appears insufficient to interfere with anesthetic binding and induce large functional effects. The rigidity, spatial orientation, and electrostatics of pCMBS in contrast to hexyl-MTS likely contribute to the disparate functional changes and anesthetic protection results.

Lacking evidence that n-alkyl-MTS modifications at α1L232C overlap with the anesthetic site in the β+/α- interface, our results cannot directly constrain the distance between α1L232 and
bound anesthetics. However, alkyl-MTS modification experiments in α1L232Wβ3M286Cy2L receptors indirectly constrain these spatial relationships.

In α1L232Wβ3M286Cy2L receptors (Figs 2 and 4) we observed an unambiguous cut-on for modifiers between methyl-MTS and ethyl-MTS and a clear cut-off between pentyl-MTS and hexyl-MTS. The modification and protection results with ethyl-MTS through pentyl-MTS in these receptors fulfilled all criteria for inferring overlap of the modified β3M286C sidechains and bound etomidate or propofol.

**FIGURE 5 HERE**

Figure 5 depicts a simple steric interpretation of results in α1L232Wβ3M286Cy2L (Fig 5A) and α1β3M286Cy2L receptors (Fig 5B). In this linear model, replacement of α1L232 with tryptophan reduces the cut-on size for alkyl-MTS modifiers by one methyl group, indicating that both etomidate and propofol bind about 1.3 Å closer to β3M286C sidechains. Additionally, the cut-off size for alkyl-MTS reagents overlapping the anesthetic site is reduced by at least one and possibly two methyl groups. The uncertainty is due to unavailability of n-heptyl-MTS, which might revise the cut-off in α1β3M286Cy2L receptors. Of note, in α1β3M286Cy2L octyl-MTS and decyl-MTS exposures produced persistent functional changes that were not blocked by either etomidate (Fantasia et al., 2021) or propofol (Fig 3). The failure of anesthetics to block effects of these large modifiers may be due to slow modifier washout from oocytes in protection trials (Fantasia et al., 2021). Fig 5B suggests that alkyl-MTS reagents larger than heptyl-MTS shouldn’t modify α1β3M286Cy2L receptors if they are extended toward α1L232. Alternatively, the larger -S-alkyl modifications of β3M286C may extend into the adjacent lipid membrane in α1β3M286Cy2L receptors but not in α1L232Wβ3M286Cy2L receptors. Indeed, cryo-EM structures of etomidate and propofol bound in GABA_A receptors (Kim et al., 2020) show βM286 and particularly αL232 sidechains oriented more toward membrane lipids than toward each other (Fig 6). Though we can only speculate about sidechain orientation, our results indicate
that the αL232W mutation sterically displaces anesthetics toward β3M286C, indicating intermolecular contact between α1L232W and bound anesthetics. Comparing Fig 5A and 5B suggests an indirect estimate of the distance between α1L232 and the anesthetic site of under 1 Å, based on the length difference between tryptophan and leucine (2.1 Å) minus the difference in distances to βM286C (1.3 Å). With correction for C-H bonds, this estimate is consistent with cryo-EM structures (Fig. 6).

FIGURE 6 HERE

Because hexyl-MTS exposure doesn’t affect α1L232Wβ3M286Cy2L receptor function we infer that it is excluded from the mutated anesthetic site. Poor reactivity is ruled out because hexyl-MTS readily modifies α1L232Cβ3y2L and α1β3M286Cy2L receptors. With this cut-off size, the linear molecular ruler model estimates that the maximal distance between the α-carbons of β3M286C and α1L232W is approximately 18 Å (Fig 5A). Correcting for mutant sidechain lengths, the maximal distance between β3M286 and α1L232 terminal sidechain carbons could be 8 Å (Fig 5C) or larger, depending on orientations. As maximal estimates, our results are consistent with cryo-EM structures (Fig 6), while likely representing different functional states.

Our inferences are based on several assumptions. First, that covalent modifications underlie persistent functional effects. Control experiments demonstrated that an alkyl-MTS reagent and an accessible engineered cysteine are both necessary to produce persistent functional changes (Fantasia et al., 2021). Another assumption is that anesthetic inhibition of persistent effects is due to steric interactions with alkyl-MTS modifiers rather than allosteric effects. Indeed, prior SCAMP studies showed that anesthetics producing similar functional effects in GABA<sub>A</sub> receptors selectively protect cysteines engineered near amino acids where photolabeling identified their binding (Nourmahnad et al., 2016; Ziemba et al., 2018). Additionally, we assume that mutations and covalent modifications minimally alter peptide backbone folding. However,
our current results combined with other evidence suggest that the transmembrane inter-subunit distances in GABA$_A$ receptors may greatly vary.

Bali et al (2009) reported that in functional GABA-bound receptors, $\beta$2M286C formed stable disulfide bonds with $\alpha$1Y225C and $\alpha$1Q229C, both located closer to the extracellular end of the $\alpha$1-M1 transmembrane helix on the same face as $\alpha$1L232, while $\alpha$1L232C formed a disulfide bond with $\beta$2F289C, about one helical turn intracellular from $\beta$2M286. Borghese et al (2014) confirmed these results. To form stable disulfide bonds, the $\alpha$-carbons of cysteine sidechains must converge to within 5.6 Å (Careaga and Falke, 1992). In cryo-EM structures of $\alpha$1$\beta$2$\gamma$2L receptors with either propofol or etomidate bound (Fig 6), the distances between $\alpha$-carbons of $\alpha$1L232 and $\beta$2M286 are about 10 Å, while the distances between $\alpha$-carbons of $\beta$3M286 and $\alpha$1Y229 or between $\alpha$-carbons of $\alpha$1L232 and $\beta$2F289 are both over 11 Å. Thus, the ability of $\alpha$1-M1 cysteine substitutions to form disulfide bonds with $\beta$3-M3 cysteine substitutions in GABA-activated receptors does not correlate with their proximity in wild-type cryo-EM structures. This might be due to altered backbone peptide folding, side-chain orientations, and electrostatics in cysteine mutants or non-physiological receptor structures in cryo-EM studies, thought to represent desensitized receptors. Assessing cryo-EM structures of mutant GABA$_A$ receptors could help resolve these uncertainties.

Considering our alkyl-MTS results together with disulfide bond results in mutated functional receptors suggests that the $\beta^+/\alpha^-$ inter-subunit clefts can expand and contract, bringing $\alpha$-carbons on opposing faces of $\beta$-M3 and $\alpha$-M1 transmembrane helices as close as 5.6 Å or as far apart as 18 Å. These distances may represent extremes that occur with low probability. However, they are reasonably consistent with recent course-grained molecular dynamics simulations (Nakata et al., 2023) based on cryo-EM structures of $\alpha$1$\beta$2$\gamma$2L GABA$_A$ receptors with anesthetics bound (Kim et al., 2020).
Our present results also indicate that steric relationships between βM286 or αL232 and etomidate vs. propofol are similar at 1.3 Å resolution, yet different. The different interactions between βM286 and propofol vs. etomidate is based on our observation that ethyl-MTS modification of α1β3M286Cγ2L enhanced etomidate modulation (Fantasia et al., 2021), but not propofol modulation (Fig 3A). This suggests that propofol binds slightly farther away than etomidate from ethyl-MTS modified βM286C, losing some gating enhancement energy due to attractive interactions. This difference between propofol and etomidate modulation is absent in ethyl-MTS modified α1L232Wβ3M286Cγ2L receptors, in which both drugs interfere with this modified cysteine. On the other side of the anesthetic site, prior studies showed that pCMBS modification of α1L232C is blocked by etomidate, but not by propofol (Nourmahnad et al, 2016). Overall, these data suggest that etomidate, which is larger than propofol, binds slightly closer than propofol to both βM286 and αL232, consistent with the cryo-EM structures of Kim et al (2020). In these, the shortest carbon-carbon distance between βM286 and etomidate is 3.3 Å while the shortest distance to propofol (carbon-oxygen) is 3.7 Å. The shortest analogous distances from α1L232 are 3.8 Å to etomidate and 4.2 Å to propofol (Fig 6).

In addition to length of modified sidechains, other biophysical features likely influence anesthetic binding. For example, hydrophobicity varies with n-alkyl-MTS size, while propofol and etomidate have distinct hydrogen-bonding abilities and electrostatic features. Nonetheless, the use of probes with incremental size differences of a single methyl group and resulting clear cut-on and cut-off effects fits well with our interpretive framework.

In summary, SCAMP studies in functional α1β3M286Cγ2L and α1L232Wβ3M286Cγ2L receptors indicate that α1L232W mutations reduce the maximal size of n-alkyl-MTS reagents that can modify β3M286C, while apparently shifting bound etomidate or propofol closer to βM286 by about 1.3 Å. We infer that both etomidate and propofol bind close to α1L232 (within 1Å). Our inferences regarding intermolecular distances in these sites in functional receptors are
consistent with cryo-EM structures, while suggesting that maximal distances between α-M1 and β-M3 transmembrane helices can be much greater than those in cryo-EM structures and can vary by more than 10 Å.
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Data Availability Statement: The authors declare that all the data supporting the findings of this study are contained within the paper.

Authorship Contributions:

*Participated in research design:* K. Bhave and S. Forman

*Conducted experiments:* K. Bhave

*Performed data analysis:* K. Bhave and S. Forman

*Wrote or contributed to writing of the manuscript:* K. Bhave and S. Forman
References:


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Figure Legends

Figure 1: Effects in α1L232Cβ3γ2L receptors following alkyl-MTS exposure without and with etomidate.  
A) Traces illustrate voltage-clamp currents from a single oocyte expressing α1L232Cβ3γ2L receptors elicited with high (3 mM, solid bars) and low (1 µM, double-line) GABA, both before (left traces) and after (right traces) 30 s exposure to 3 mM GABA + 300 µM hexyl-MTS (9 mM x s). The Low:high GABA response ratio increases from 0.018 to 0.038 following hexyl-MTS exposure, resulting in a modification ratio of 2.2.  
B) Low:high GABA modification ratios (mean ± sd; n = 5 each) following GABA + 9 mM x s alkyl-MTS exposures. Statistical comparisons to GABA control exposure results (n = 40) were calculated using one-way ANOVA with Dunnett’s tests. Only pentyl-MTS and hexyl-MTS exposures produced statistically significant effects.  
C) Low:high GABA modification ratios increase with hexyl-MTS exposure, reaching a maximum (2.4 ± 0.31; mean ± sd, n = 7) at 9 mM x s and above.  
D) Addition of etomidate (300 µM) during 9 mM x s hexyl-MTS exposure did not alter modification effects. The inset in panel D depicts the chemical structure of R-etomidate.

Figure 2: Effects in α1L232Wβ3M286Cγ2L receptors following alkyl-MTS exposure without and with etomidate.  
A) Traces illustrate voltage-clamp currents from a single oocyte expressing α1L232Wβ3M286Cγ2L receptors elicited with high (10 mM, solid black bar) and low (1 µM, black double-line) GABA followed by low GABA + etomidate (300 µM, red bar), both before (left traces) and after (right traces) 30 s exposure to 10 mM GABA + 300 µM hexyl-MTS (9 mM x s). The low:high GABA response ratio is 0.019 before hexyl-MTS exposure and 0.023 after, resulting in a modification ratio of 1.2. The etomidate enhancement ratio is 3.9 before hexyl-MTS exposure and 3.8 after, resulting in a modification ratio of 0.97.  
B) Traces illustrate voltage-clamp currents from a single oocyte expressing α1L232Wβ3M286Cγ2L receptors studied as in panel A, before and after exposure to pentyl-MTS (9 mM x s). The low:high GABA
response ratio is 0.018 before hexyl-MTS exposure and 0.108 after, resulting in a modification ratio of 6.0. The etomidate enhancement ratio is 6.3 before pentyl-MTS exposure and 1.7 after, resulting in a modification ratio of 0.27. C) Traces illustrate voltage-clamp currents from a single oocyte expressing α1L232Wβ3M286Cγ2L receptors studied as in panel B, but with addition of 300 µM etomidate during pentyl-MTS exposure. The low:high GABA response ratio is 0.010 before hexyl-MTS exposure and 0.017 after, resulting in a modification ratio of 1.7. D) Scatter plots summarizing low:high GABA modification ratios following exposure to 10 mM GABA + eight alkyl-MTS reagents (9 mM x s; n = 5 each). Bars overlaying data points represent mean ± sd. Statistical comparisons to control (n = 40) were analyzed using one-way ANOVA with Dunnett’s tests. The horizontal dotted line represents mean control. Red dashed vertical lines identify cut-on and cut-off for statistically significant increases from control. E) Scatter plots summarizing etomidate enhancement modification ratios following exposure to 10 mM GABA + seven alkyl-MTS reagents (9 mM x s; n = 5 each). Bars overlaying data points represent mean ± sd. Statistical comparisons to control (n = 35) were analyzed using one-way ANOVA with Dunnett’s tests. The horizontal dotted line represents mean control. Red dashed vertical lines identify cut-on and cut-off for statistically significant decreases from control. F) Summary of low:high GABA modification ratios (mean ± sd; n = 5 each) in oocytes expressing α1L232Wβ3M286Cγ2L receptors exposed to GABA + alkyl-MTS modifiers (9 mM x s) without (black columns) or with 300 µM etomidate (red columns). Statistical comparisons were performed using unpaired Student’s t-tests. ETO = etomidate.

Figure 3: Propofol effects in α1β3M286Cγ2L receptors exposed to alkyl-MTS reagents. A) A scatter plot summarizing propofol enhancement modification ratios in oocytes expressing α1β3M286Cγ2L receptors following exposure to 3 mM GABA + seven alkyl-MTS reagents (9 mM x s; n = 5 each). Bars overlaying data points represent mean ± sd. Statistical comparisons
to controls (no alkyl-MTS reagent; n = 35) were analyzed using one-way ANOVA with Dunnett’s tests. The horizontal dotted line represents the mean control value. The red dashed vertical line indicates the apparent cut-on between ethyl-MTS and propyl-MTS. The inset in panel A depicts the chemical structure of propofol. B) Summary (mean ± sd; n = 5 each) of low:high GABA modification ratios in oocytes expressing α1β3M286Cγ2L receptors exposed to GABA + five alkyl-MTS modifiers (9 mM x s) without (black columns) or with 100 µM propofol (white columns). Statistical comparisons were performed using unpaired Student’s t-tests. PRO = propofol.

**Figure 4: Propofol effects in α1L232Wβ3M286Cγ2L receptors exposed to alkyl-MTS reagents.** A) A scatter plot summarizing propofol enhancement modification ratios in oocytes expressing α1L232Wβ3M286Cγ2L receptors following exposure to 10 mM GABA + seven alkyl-MTS reagents (9 mM x s; n = 5 each). Bars overlaying data points represent mean ± sd. Statistical comparisons to controls (no alkyl-MTS; n = 35) were analyzed using one-way ANOVA with Dunnett’s tests. The horizontal dotted line represents the mean control value. The red dashed vertical lines indicate the apparent cut-on between methyl-MTS and ethyl-MTS and the cut-off between pentyl-MTS and hexyl-MTS. B) Summary (mean ± sd; n = 5 each) of low:high GABA modification ratios in oocytes expressing α1L232Wβ3M286Cγ2L receptors exposed to GABA + alkyl-MTS modifiers (9 mM x s) without (black columns) or with 100 µM propofol (white columns). Statistical comparisons were performed using unpaired Student’s t-tests.

**Figure 5: Spatial interpretation of alkyl-MTS modification and anesthetic protection results.** A) Depiction of alkyl-MTS modification results in α1L232Wβ3M286Cγ2L receptors. Modified β3M286C sidechains that overlap with bound etomidate or propofol are shaded red, showing a cut-on between methyl-MTS and ethyl-MTS and a cut-off beyond pentyl-MTS. Vertical black lines represent intervals of 1.3 Å, the incremental alkyl chain-length produced by
addition of one methyl group. The gap between the sidechain and α1L232W represents carbon-hydrogen bonds (1.1 to 2.2 Å). **B)** Depiction of alkyl-MTS modification results in α1β3M286Cy2L receptors. Modified β3M286C sidechains that overlap with bound anesthetics are shaded red, showing a cut-on between ethyl-MTS and propyl-MTS and a cut-off beyond hexyl-MTS. **C)** Assuming minimal changes in peptide backbone configuration, the maximal distance between β3M286 and α1L232, based on the estimated maximal distance between α-carbons (19 Å from panel A) minus side-chain lengths with hydrogens (6.3 Å for methionine and 4.7 Å for leucine) is 8 Å.

**Figure 6:** Cryo-electron micrography structures of anesthetics bound to α1β2γ2L receptors. *Structures were reported by Kim et al (2020).*  
A) The protein databank (PDB) structure 6X3V with etomidate bound was imaged using UCSF Chimera. This image depicts one of the two β+/α- intersubunit transmembrane sites where etomidate binds, viewed from the surrounding lipid. Transmembrane helical backbones are shown as ribbons, with color coding β = blue and α = yellow. Sidechains of interest are labeled and depicted in ball and stick form with color-coding by heteroatom (without hydrogens): carbon = gray; oxygen = red; nitrogen = blue; and sulfur = yellow. Etomidate is depicted in ball and stick form with all atoms colored magenta. Distances between the α-carbon of βM286C and those of αQ229, αL232, and αM236 as well as the shortest carbon-carbon distances from etomidate to βM286 and αL232 are shown as dashed lines and labeled with lengths in angstroms (Å). B) A similar view of PDB 6X3T depicting propofol (colored peach) bound within the same site. Minimal distances shown from propofol to βM286 and αL232 are from the oxygen of propofol.
Figure 1

A) shows a comparison of GABA and Hexyl-MTS effects. B) displays a bar graph with low-high GABA modification ratios for different MTS derivatives. C) illustrates a plot of low-high GABA modification ratio against Hexyl-MTS concentration. D) presents a comparison of GABA effects with and without Hexyl-MTS and etomidate.
Figure 5

βM286C + Alkyl-MTS

A

C
S
S
C
C
C
C

B

C
S
S
C
C
C
C

C

5.3 Å

C
C
S
C

C
C
C

6.0 Å

C

10.7 Å

C
C
C
C
C
C

3.9 Å

C
C
C

αL232W