Alphaxalone Binds in Inner Transmembrane β^+–α^- Interfaces of $\alpha1\beta3\gamma2$ γ-Aminobutyric Acid Type A Receptors

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ABSTRACT

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

Methods: The authors created 12 single-residue β3 cysteine mutations: β3T262C and β3T266C in β3-M2; and β3M283C, β3Y284C, β3M286C, β3G287C, β3F289C, β3V290C, β3F293C, β3L297C, β3E298C, and β3F301C in β3-M3 helices. The authors coexpressed α1 and γ2L with each mutant β3 subunit in *Xenopus* oocytes and electrophysiologically tested each mutant for covalent sulfhydryl modification by the water-soluble reagent para-chloromercuribenzenesulfonate. Then, the authors assessed whether receptor-bound alphaxalone, etomidate, or propofol blocked cysteine modification, implying steric hindrance.

Results: Eleven mutant β3 subunits, when coexpressed with α1 and γ2L, formed functional channels that displayed varied sensitivities to the three anesthetics. Exposure to para-chloromercuribenzenesulfonate produced irreversible functional changes in ten mutant receptors. Protection by alphaxalone was observed in receptors with β3V290C, β3F293C, β3L297C, or β3F301C mutations. Both etomidate and propofol protected receptors with β3M286C or β3V290C mutations. Etomidate also protected β3F289C. In $\alpha1\beta3\gamma2L$ structural homology models, all these protected residues are located in transmembrane β^+–α^- interfaces.

Conclusions: Alphaxalone binds in transmembrane β^+–α^- pockets of synaptic $GABA_A$ receptors that are adjacent and intracellular to sites for the potent anesthetics etomidate and propofol. **(Anesthesiology 2018; 128:338-51)**

NEUROSTEROIDS (neuroactive steroids), including the general anesthetic alphaxalone (ALX), allopregnanolone, and tetrahydro-deoxycorticosterone, are potent rapid-acting anxiolytics, anticonvulsants, amnestics, and sedative-hypnotics.[1] These effects are linked to enhanced gating of γ-aminobutyric acid type A ($GABA_A$) receptors, the main inhibitory neurotransmitter receptors in mammalian brain and major molecular targets for the general anesthetics propofol and etomidate.[2,3] Typical synaptic $GABA_A$ receptors consist of 2α, 2β, and 1γ subunits arranged $\beta\alpha\beta\alpha\gamma$ counterclockwise, viewed from the extracellular space.[4] Each $GABA_A$ subunit contains an N-terminal extracellular domain and a transmembrane domain with four α helices: M1 to M4. Five M2 helices surround a receptor's central chloride channel, while M1 and M3 helices form an intermediate ring between M2 and M4 helices. Subunit interfaces are designated β^+–α^- (two per receptor), α^+–β^-, γ^+–β^-, and α^+–γ^-, where + corresponds to the M3 face and – is the M1 face.

Data locating neurosteroid sites on $GABA_A$ receptors are sparse and inconsistent (table 1).[5–22] Pharmacokinetic studies indicate that neurosteroids reach $GABA_A$ receptors *via* membrane lipids.[23] Mutations in α1-M1 at α1M236, α1T237, and α1I239 reduce neurosteroid sensitivity.[5,13] These residues map to outer transmembrane β^+–α^- clefts in

What We Already Know about This Topic

- Alphaxalone and related endogenous or exogenous neurosteroids are potent anxiolytics, anticonvulsants, amnestics, and sedative-hypnotics.
- The pharmacologic effects of neurosteroids, like those of propofol and etomidate, are linked to enhanced γ-aminobutyric acid type A ($GABA_A$) receptor gating. However, the sites within $GABA_A$ receptors that bind to the neurosteroids are not clearly defined.

What This Article Tells Us That Is New

- Alphaxalone contacts were identified in the inner transmembrane β^+–α^- intersubunit clefts of γ-aminobutyric acid type A ($GABA_A$) receptors. These sites are adjacent to the outer transmembrane sites where etomidate and propofol act.
- The results suggest that large portions of the transmembrane intersubunit clefts of $GABA_A$ receptors are allosterically coupled to ion channel gating. These clefts form a number of distinct binding sites for pharmacologic agents that include neurosteroids and currently used intravenous anesthetics.

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homology models based on glutamate-gated chloride (GluCl) channels from *Caenorhabditis elegans*[24] (fig. 1)[25] and are identified by photolabeling and substituted cysteine modification-protection (SCAMP) studies as contacts for etomidate and propofol (table 1).[26] Ivermectin binds to outer transmembrane intersubunit pockets on GluCl[24] and triiodothyronine displaces both ivermectin and allopregnanolone from homologous $GABA_A$ receptor sites, including the etomidate/propofol sites.[27] Thus, neurosteroids may act through the outer transmembrane β^+–α^- pockets where etomidate and propofol bind.

Other evidence indicates that neurosteroid sites are separate from etomidate and propofol sites. Neurosteroids synergize with etomidate and its derivatives when coapplied to $GABA_A$ receptors.[28,29] Previous SCAMP experiments find no ALX interactions at several etomidate and propofol contacts in outer transmembrane β^+–α^- clefts or other homologous pockets in $\alpha1\beta3\gamma2L$ receptors.[5,21] Other evidence points to inner transmembrane β^+–α^- neurosteroid sites. Mutations in inner α1-M1 at α1Q242 reduce neurosteroid sensitivity.[13,14] The photolabel (3α,5β)-6-azipregnanolone (6-AziP) incorporates in inner β3-M3 at β3F301, but this study used β3 homomeric receptors.[22] Finally, β2Y284 mutations also impair neurosteroid effects.[13] This residue's location in β3 crystals[30] and homology models (fig. 1) suggests neurosteroid sites within β3 intrasubunit helix bundles.

To test whether ALX binds in β^+–α^- transmembrane clefts and to compare ALX sites to those for etomidate and propofol, we used SCAMP to assess drug contacts on β3-M2 and β3-M3 helices in $\alpha1\beta3\gamma2L$ receptors. Using the structure of β3 homomeric receptors[30] and our GluCl-based structural homology model[25] (fig. 1), we selected residues spanning most of the β3-M3 helix, from β3M283 (outer) to β3F301 (inner), most facing the β^+–α^- interface, and several facing the intrasubunit β3 helix pocket. Our results suggest that ALX contacts β3-M3 at β^+–α^- interfacial residues that are adjacent and intracellular to those for propofol and etomidate.

Materials and Methods

Animals

Oocytes were harvested from female *Xenopus laevis* frogs in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, Maryland). Animal use in this study was approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee (Boston, Massachusetts; protocol No. 2005N000051). Frogs were housed and maintained in a veterinarian-supervised facility and anesthetized in tricaine during oocyte collection. All efforts were made to minimize suffering.

Materials

Alphaxalone was purchased from Tocris Bioscience (United Kingdom) and propofol (2,6-diisopropylphenol)

Table 1. Evidence of Neurosteroid and Anesthetic Contacts in β^+–α^- Transmembrane Interfaces of $GABA_A$ Receptors

Residue	Receptor	Mutant Effects*	Photolabels†	SCAMP‡
α1L232	α1β3γ2L	ETO, PRO[5]	—	ETO[5,6]
α1M236	α1β3γ2L	ETO, PRO, ALX[5,7]	Azi-ETO[8]	ETO, PRO[5,6,9]
	α1β3		Azi-ETO[10]	
	α1β3		TDBzl-ETO[11]	
	α1β3		Azi-Pm[12]	
α1T237	α1β2γ2L	Neurosteroids[13]		
	α1β3γ2L		—	ETO[6]
α1I239	α1β2γ2L	Neurosteroids[13]		
	α1β3γ2L		—	—[6]§
	α1β3		Azi-Pm[12]	
α1Q242	α1β2γ2L	Neurosteroids[13,14]		
	α1β3γ2L		—	—[6]§
β3N265	α1β3γ2L	ETO, PRO[15–18]	—	ETO, PRO[9]
β3M286	α1β2/3γ2	ETO, PRO[7,18,19]	Azi-ETO[8,10]	ETO, PRO[20,21]
	α1β3		TD-Bzl-ETO[11]	
	α1β3		Azi-Pm [12]	
β3F289	α1β3γ2L		(ETO)[8]	
β3V290	α1β3		TD-Bzl-ETO[11]	
β3F301	β3		6-AziP[22]	

Neurosteroids are allopregnanolone (ALLOP) and tetrahydro-deoxycorticosterone (THDOC). — indicates negative results.

*Drugs displaying reduced enhancement of submaximal GABA responses in mutant receptors are listed. Not all loci have been tested with ETO, PRO, and ALX. Negative effects of α1M236 and βM286 mutations on ALX sensitivity have been reported.[5,18]†Direct or indirect (indicated by parentheses) photolabeling evidence is included. Specifically, β3F289 photolabeling by m-trifluoromethyl-mephobarbital is inhibited by etomidate.‡Drugs demonstrating protection are listed. Not all loci have been tested with ETO, PRO, and ALX. Negative results have been reported for PRO at α1L232C and ALX at α1L232C, α1M236C and β3M286C.[5,6,21]§Application of a cysteine modifying reagent (*p*-chloromercuribenzenesulfonate) to α1I239C and α1Q242C did not alter function, precluding protection studies.

6-AziP = 6-azi-pregnanolone; ALX = alphaxalone; Azi-ETO = azi-etomidate; Azi-Pm = *m*-azi-propofol; ETO = etomidate; GABA = γ-aminobutyric acid; $GABA_A$ = γ-aminobutyric acid type A; PRO = propofol; SCAMP = substituted cysteine modification-protection; TD-Bzl-ETO = *p*-trifluoromethyldiazirylphenyl-etomidate.

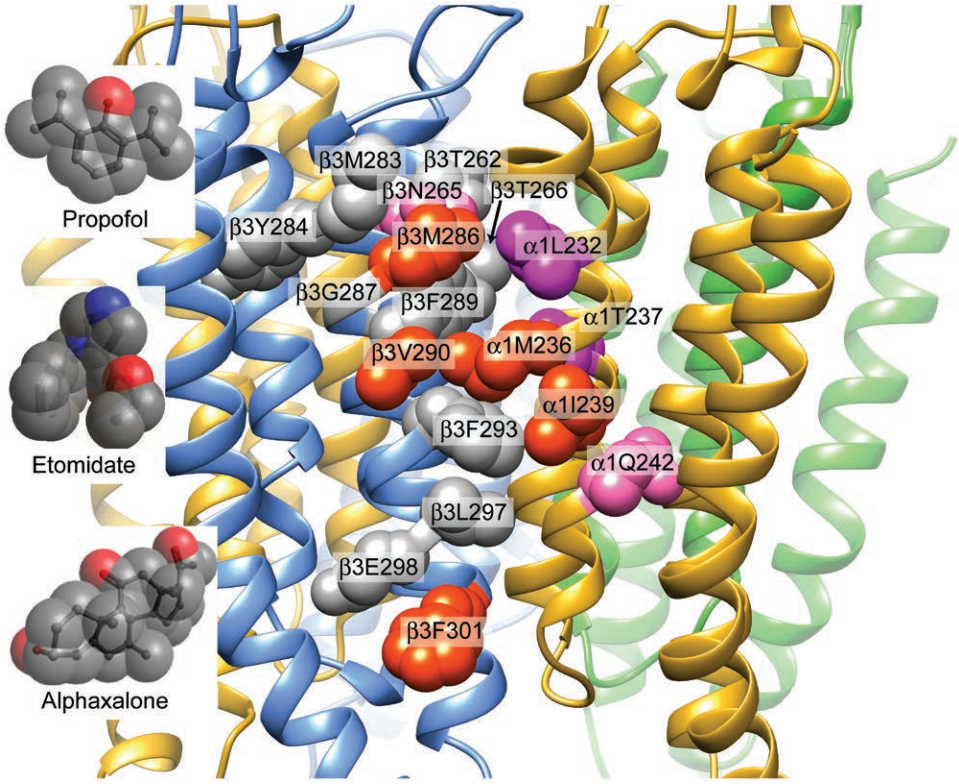

Fig. 1. General anesthetic contacts within the γ-aminobutyric acid type A ($GABA_A$) receptor β^+–α^- transmembrane cleft. The transmembrane domain of a α1β3γ2L structural homology model based on GluCl (Protein Data Bank 4COF) is depicted.[25] Subunit peptide backbones are shown as ribbons (α1 = *yellow*; β3 = *blue*; γ2L = *green*), with sidechains of interest (table 1) shown in space-filling mode and labeled. Amino acid sidechains on β3-M3 and α1-M1 that are directly photolabeled by analogs of one or more study anesthetics are colored *orange-red*. Anesthetic contact sidechains that have previously been identified using substituted cysteine modification-protection are colored *purple*. Other β3-M2 and β3-M3 sidechains that line the β^+–α^- cleft, and three sidechains predicted to face the β3 intrasubunit helix bundle pocket (Y284, G287, and E298), are colored *gray*. The location of α1Q242 (*pink*) is also shown. *Inserts* display the molecular space-filling structures of propofol, etomidate, and alphaxalone, approximately scaled to the receptor model. Hydrogens have been hidden for clarity.

was purchased from Sigma-Aldrich (USA). Both were stored as 10 mM solutions in dimethyl sulfoxide (DMSO) and diluted in electrophysiology buffer for experiments. R-Etomidate was purchased from Hospira, Inc. (USA) as a 2 mg/ml (~8.2 mM) solution in 35% propylene glycol:water and diluted in electrophysiology buffer for experiments. We have previously shown that DMSO and propylene glycol at the dilutions used during electrophysiology experiments produce no effects on $GABA_A$ receptor function.[25] R-mTFD-MPAB (R-allyl-m-trifluoromethyl-mephobarbital)[31] was stored as a 100 mM solution in DMSO and diluted in electrophysiology buffer for experiments. Para-chloromercuribenzenesulfonic acid sodium salt (pCMBS) was purchased from Toronto Research Chemicals (Canada). Fresh pCMBS stock solutions in electrophysiology buffer were prepared on the day of use and kept on ice until final dilution. γ-Aminobutyric acid (GABA), picrotoxin, salts, and buffers were purchased from Sigma-Aldrich.

$GABA_A$ Receptor Expression in *Xenopus* Oocytes. Oocytes were prepared for use as previously described.[5] Complementary DNAs encoding human α1, β3, and γ2L $GABA_A$ receptor subunits in pCDNA3.1 expression vectors (Thermo Fisher Scientific, USA) were used. Cysteine mutations were introduced into β3 by site-directed mutagenesis using QuikChange kits (Agilent Technologies, USA). After sequencing several clones through the entire coding region, one clone for each mutant was chosen for further use. Messenger RNAs were synthesized on linearized DNA templates using mMessage mMachine kits (Thermo Fisher Scientific), purified, and combined at ratios of 1α:1β:5γ (final concentration 1 ng/nl in RNAase-free water). Oocytes were injected with ~50 ng mRNA mix and incubated in ND96 buffer (in mM: 96 NaCl, 2 KCl, 1 $CaCl_2$, 0.8 $MgCl_2$, 10 HEPES, pH 7.5) supplemented with ciprofloxacin (2 mg/ml) and amikacin (100 μg/ml) at 17°C for 48 to 72 h before electrophysiologic studies.

Two Electrode Voltage-clamp Electrophysiology. Electrophysiologic experiments were performed in ND96 buffer at 21 to 23°C as previously described.[5] Oocytes were placed in a 30 μl custom flow-cell, impaled with borosilicate glass microelectrodes filled with 3 M KCl (resistance < 1 MΩ), then voltage-clamped at –50 mV (model OC-725C, Warner Instruments, USA). Superfusion solutions in ND96 were controlled by electrical valves (VC-8, Warner Instruments) and delivered

at a rate of 2 to 3 ml/min from glass reservoir syringes *via* polytetrafluoroethylene tubing and a polytetrafluoroethylene micromanifold (MP-8, Warner Instruments). Specialized software and a digital input/output interface (pClamp 8.0 and Digidata 1322, both from Molecular Devices, USA) were used to coordinate delivery of solutions and recordings. Current signals were filtered at 1 kHz, digitized at 100 Hz, and stored on a computer disk for offline analysis.

GABA Concentration-responses, Spontaneous Receptor Activity, and GABA Efficacy. Each mutant receptor was initially characterized to establish its sensitivity to GABA, maximal GABA efficacy, and whether it was spontaneously active. Voltage-clamped oocytes were exposed to GABA solutions (range: 0.1 μM to 10 mM) for 10 to 20 s, followed by 5 min ND96 wash. Normalization sweeps at the maximum GABA concentration for the specific receptor (greater than 10 × EC_{50}; 1 to 10 mM) were recorded every second or third experiment. At least three oocytes from two different frogs were used for each concentration-response.

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

Maximal GABA efficacy for each receptor was estimated by comparing peak currents elicited with maximal GABA (1 to 10 mM) to currents elicited with high GABA supplemented with either 2.5 to 5 μM ALX or 3.2 to 6.4 μM etomidate, depending on the receptor's drug sensitivity (see GABA EC5 Enhancement section below). Agonist efficacy was calculated by normalizing maximal GABA responses to GABA + anesthetic responses in the same cell, assuming the latter represents 100% activation (N ≥ 3 cells).

GABA EC5 Enhancement. Each mutant was also characterized for sensitivity to etomidate, propofol, and ALX. Voltage-clamped oocytes expressing $GABA_A$ receptors were repetitively exposed for 20 s to GABA EC5 (eliciting ~5% of maximal GABA response) separated by 5 min wash until three stable responses (varying by less than 5%) were sequentially recorded. The oocyte was then exposed to anesthetic for 30 s, followed by 20 s exposure to a solution containing GABA EC5, combined with anesthetic at 2 × EC50 for loss-of-righting-reflexes in tadpoles: 2.5 μM alphaxalone,[32] 3.2 μM etomidate,[33] or 5 μM propofol.[34] For each receptor type and three anesthetics, multiple measurements of current response to GABA EC5 and GABA EC5 + anesthetic were obtained in at least four oocytes from two different frogs. EC5 enhancement (mean ± SEM; N ≥ 4) was calculated from the set of individual oocyte ratios of currents measured with anesthetic present to EC5 GABA alone.

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

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

Protection experiments were performed in the presence of maximally activating GABA, as previously described,[5] so control modification conditions were pCMBS + GABA. Oocytes were exposed to anesthetic for 30 s followed by exposure to a solution of pCMBS + GABA + anesthetic. Postmodification wash and response tests were identical to control modification conditions (*i.e.*, usually with no anesthetic present, but see below in this section). Anesthetic concentrations used in initial protection studies were chosen to maximize site occupancy, while enabling washout within 5 min (10 μM etomidate, 20 μM propofol, and 10 μM ALX). In receptors with β3F289, β3F293C, and β3L297C mutations, higher concentrations of anesthetics (50 μM etomidate, 100 μM propofol, or 50 μM ALX) were also used in protection experiments. Under these conditions, anesthetic washout between pCMBS exposure and testing for modification effects was extremely slow. Therefore, we used

an alternative approach to low GABA responses, measuring direct activation by anesthetics alone (50 μM etomidate, 100 μM propofol, or 50 μM ALX), normalized to high GABA responses. At least two anesthetics were tested in the same manner, to test for drug-specific interactions. In the case of receptors with β3V290C mutations, we tested for allosteric effects (*i.e.*, whether all anesthetics affect pCMBS modification similarly), by including SCAMP studies with 10 μM mTFD-MPAB, a barbiturate hypnotic that acts through $GABA_A$ receptor sites outside the β^+–α^- interfaces.[8,31] For each cysteine mutant, at least five oocytes were studied in control modification experiments and at least four oocytes were studied in each set of anesthetic protection experiments. Group sample sizes of five per group were based both on prior experience and a power analysis performed as previously described,[5] using a one-tail Student's *t* test with α = 0.017 (adjusted for three drug comparisons to each control).

Data Analysis and Statistics

Results in text and figures are mean ± SEM unless otherwise indicated.

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

$$I_{norm} = (I_{max} - I_{min}) / (1 + 10^{(LogEC50 - Log[GABA] \times nH)}) + I_{min} \quad (1)$$

where EC_{50} is the half-maximal activating GABA concentration, and nH is the Hill slope. Mean GABA EC_{50} and 95% CI are reported. To assess whether mutations altered GABA EC_{50} relative to wild-type, we performed sum-of-squares F-tests in GraphPad Prism 5.02, using $P < 0.0045$ as a statistical significance threshold (the Bonferroni correction for $P < 0.05$ with 11 comparisons).

Functional Characteristics of Mutant Receptors. To test whether mutations altered spontaneous activity and/or GABA efficacy from wild-type values, we used one-way ANOVA with *post hoc* Dunnett's tests (in GraphPad Prism 5.02). To test whether mutations affected receptor sensitivities to etomidate, propofol, or ALX, EC5 enhancement data for the three equipotent anesthetic concentrations in wild-type and all functional cysteine mutants was tabulated and analyzed with two-way ANOVA and Bonferroni posttests for wild-type *versus* mutation for each anesthetic (GraphPad Prism 5.02).

SCAMP. Inferences regarding contact between receptor-bound anesthetics and substituted cysteine sidechains were made when an anesthetic inhibited pCMBS modification selectively, with at least one other anesthetic failing to inhibit modification. Apparent pCMBS modification rates were calculated from data for individual oocytes expressing cysteine mutants. Either normalized maximal GABA responses (for α1β3T262Cγ2L) or normalized low:high GABA response ratios (all other mutants) were plotted against cumulative pCMBS exposure (M × s) and fitted by linear least squares with *y*-axis intercepts fixed at 1.0. The linear slope, under conditions of partial modification, is presumed to be proportional to the bimolecular reaction rate between pCMBS and the substituted cysteine sulfhydryl.

For α1β3T262Cγ2L data, apparent modification rates were calculated as the absolute values of the negative fitted slopes. Absolute slopes less than 10 $M^{-1}s^{-1}$ (the lower limit of detection) were assigned a rate of 10 $M^{-1}s^{-1}$ for statistical analysis. To identify anesthetics that either accelerated or inhibited modification of each substituted cysteine, apparent rates from control and anesthetic protection studies for that mutant were log transformed, tabulated, and compared using one-way ANOVA (GraphPad Prism 5.02) with $P < 0.05$ as a significance threshold.

Results

Functional Characteristics of β3 Cysteine Mutants

Based on both crystallographic data for β3 homomeric $GABA_A$ receptors (Protein Data Bank 4COF)[30] and our α1β3γ2L structural homology model based on GluCl bound to ivermectin (Protein Data Bank 3RHW; fig. 1),[25,26] we identified nine β3-M2 and M3 helix residues facing the β^+–α^- cleft: T262, T266, M283, M286, F289, V290, F293, L297, and F301. We created mutant β3 cDNAs encoding cysteine substitutions at these positions, as well as at Y284, G287, and E298, which are predicted to instead face the intrasubunit β3 helix-bundle pocket. Wild-type and mutant β3 subunits were coexpressed with wild-type α1 and γ2L subunits in *Xenopus* oocytes, and functionally characterized using two-microelectrode voltage-clamp electrophysiology. No GABA-activated currents were detected when β3 subunits with Y284C mutations were coexpressed with α1 and γ2L, which was consistent with prior reports.[35] All other mutations produced GABA-sensitive ion channels with sufficient oocyte currents elicited by 1 to 10 mM GABA (greater than or equal to 0.5 μA at –50 mV) for further experiments. Table 2 summarizes GABA EC_{50}, spontaneous activation, apparent maximal GABA efficacy, and the effect of pCMBS application in these mutant receptors, in comparison to wild-type α1β3γ2L. Six mutations (β3T266C, β3M286C, β3G287C, β3F293C, β3L297C, and β3E298C) significantly increased GABA EC_{50}, and one (β3F289C) reduced GABA EC_{50} approximately fivefold. Four mutant receptors characterized by increased GABA EC_{50} also exhibited significantly reduced GABA efficacy (β3M286C, β3F293C, β3L297C, and β3E298C). Like other mutations that sensitize receptors to GABA,[7,36] β3F289C was associated with both high GABA efficacy and measurable spontaneous activation. Our observations were also consistent with previous studies of β2M286C, β2G287C, and β2F289C mutations.[20,21,35,37,38]

Table 2. Functional and Pharmacologic Characteristics of α1β3γ2L $GABA_A$ Receptors with β3 Cysteine Substitutions

Receptor Type	GABA EC_{50}(μM) [95% CI] (n)	GABA Efficacy mean ± SD (n)	Spontaneous Activation mean ± SE (n)	Maximal Effect of pCMBS Modification (range)
α1β3γ2L	31 [23 to 41] (8)	0.88 ± 0.025 (5)	< 0.005 (5)	No effect
α1β3T262Cγ2L	21 [18 to 25] (4)	0.93 ± 0.03 (4)	< 0.005 (3)	Reduce max current (95–99%)
α1β3T266Cγ2L	143 [130 to 157] (6)‡	0.88 + 0.02 (4)	< 0.005 (3)	↑ low/high GABA response (10- to 13-fold)
α1β3M283Cγ2L	46 [43 to 50] (3)	0.92 ± 0.023 (3)	< 0.005 (3)	No effect
α1β3M286Cγ2L	148 [122 to 180] (3)‡	0.65 ± 0.023 (3)†	< 0.005 (3)	↑ low/high GABA response (5.8- to 7.8-fold)
α1β3G287Cγ2L	78 [67 to 92] (4)†	0.96 ± 0.034 (4)*	< 0.005 (3)	↑ low/high GABA response (2.3- to 3.5-fold)
α1β3F289Cγ2L	5.9 [5.3 to 6.7] (4)‡	0.99 ± 0.02 (4)†	0.034 ± 0.016* (4)	↑ low/high GABA response (2.8- to 4.0-fold)
α1β3V290Cγ2L	36 [32 to 41] (4)	0.92 ± 0.03 (4)	< 0.005 (3)	↑ low/high GABA response (2.3- to 3.1-fold)
α1β3F293Cγ2L	181 [143 to 229] (3)‡	0.16 ± 0.018 (3)‡	< 0.005 (3)	↑ low/high GABA response (2.6- to 4.3-fold)
α1β3L297Cγ2L	114 [104 to 125] (4)‡	0.54 ± 0.07 (5)‡	< 0.005 (4)	↑ low/high GABA response (5.5- to 7.2-fold)
α1β3E298Cγ2L	103 [88 to 112] (4)‡	0.63 ± 0.07 (3)‡	< 0.005 (3)	↑ low/high GABA response (3.5- to 5.5-fold)
α1β3F301Cγ2L	34 [30 to 39] (4)	0.93 ± 0.04 (4)	< 0.005 (4)	↑ low/high GABA response (3.8- to 4.9-fold)

Differs from wild-type at: *$P < 0.0045$, †$P < 0.0005$, ‡$P < 0.0001$, ↑ indicates an increase in the ratio of low GABA versus high GABA responses.
GABA = γ-aminobutyric acid; $GABA_A$ = γ-aminobutyric acid type A.

Anesthetic Sensitivities of Cysteine Mutants

$GABA_A$ receptor mutations may alter anesthetic modulation, which can in turn affect the conditions appropriate for SCAMP tests for drug contacts. We therefore characterized each mutant receptor's sensitivity to etomidate, propofol, and ALX by measuring anesthetic enhancement of activation by EC5 GABA. Results are summarized in figure 2. Drug solutions of 3.2 μM etomidate, 5 μM propofol, and 2.5 μM ALX are all twice the EC50 for tadpole loss-of-righting reflexes, and also similarly enhance the gating of wild-type α1β3γ2L $GABA_A$ receptors activated with EC5 GABA[5] (fig. 2). Compared to wild-type, two mutations, β3M286C and β3F289C, reduced EC5 enhancement by 3.2 μM etomidate, while β3F293C, β3L297C, and β3E298C increased EC5 enhancement by etomidate. EC5 enhancement by 5 μM propofol was also reduced by β3M286C and β3F289C, as well as by β3F293C. EC5 enhancement by 2.5 μM ALX was reduced by β3F289C, β3F293C, and β3L297C.

Effects of pCMBS on Cysteine Mutant Function

To establish conditions for SCAMP experiments, we examined the effects of pCMBS exposure, both alone and coapplied with GABA, in each of the cysteine mutants. Wild-type α1β3γ2L receptors were unaffected by pCMBS exposure at 1 mM for 60 s (N = 4). In all but one (β3M283C) of the functional cysteine-substituted mutant receptors we studied, exposure to pCMBS alone or with maximally-activating GABA concentrations induced consistent irreversible functional changes that significantly differed from repeated baseline GABA responses before pCMBS exposure (fig. 3A–I; table 2). In α1β3T262Cγ2L receptors, pCMBS exposure similarly reduced activation by both low and high GABA (fig. 3A). In the other mutant receptors, pCMBS exposure enhanced GABA sensitivity, increasing low:high response ratios in the range of twofold to 13-fold (table 2). With the exception of β3G287C, modification in the presence of GABA required lower pCMBS concentrations than without GABA at all substituted cysteines, resulting in faster apparent modification rates (fig. 3J). Results in α1β3M286Cγ2L receptors (currents not shown in fig. 3) were consistent with earlier studies of α1β2M286Cγ2L.[20,21]

Anesthetic Protection (SCAMP) with Etomidate, Propofol, and ALX

We previously have shown that SCAMP reliably identifies anesthetic contacts when drugs significantly and selectively inhibit pCMBS modification.[5] Thus, apparent initial rates of cysteine modification in control conditions (pCMBS + GABA) were compared to rates in the presence of added ALX, etomidate, or propofol in each of the modifiable mutant receptors. We chose control pCMBS modification conditions in the presence of maximally activating GABA because: (1) GABA enhances anesthetic binding and thus site occupancy; (2) GABA accelerates pCMBS modification (fig. 3J); and (3) GABA helps to establish similar mixtures

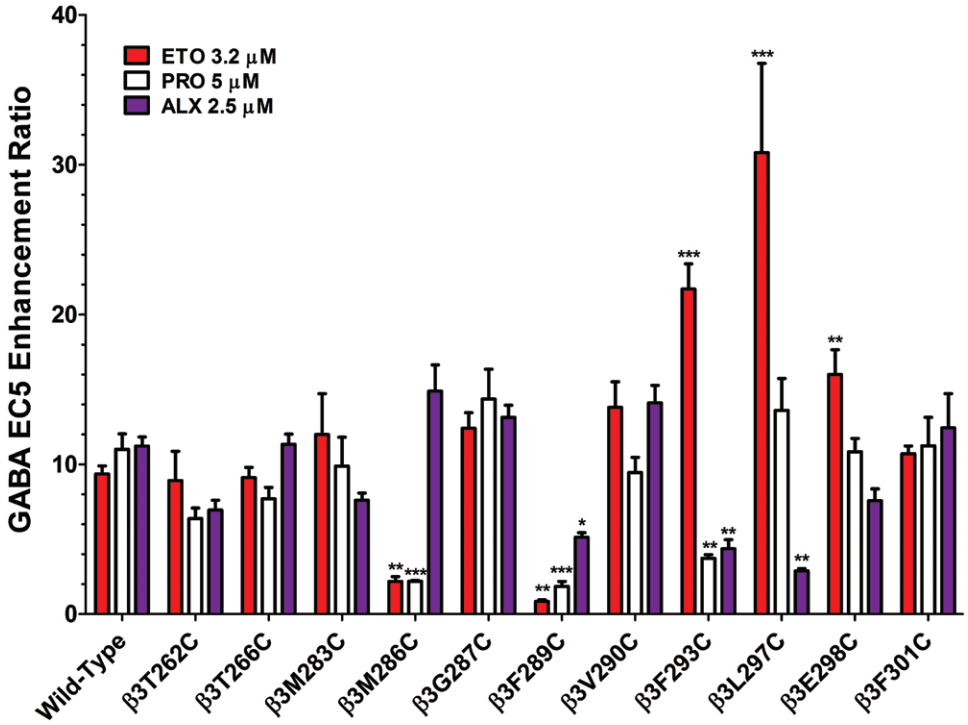

Fig. 2. Modulation of wild-type *versus* cysteine-substituted γ-aminobutyric acid type A ($GABA_A$) receptors by etomidate (ETO), propofol (PRO), and alphaxalone (ALX). Each bar represents mean ± SEM results (N ≥ 4) of experiments quantifying the anesthetic enhancement of γ-aminobutyric acid (GABA) EC5 responses in wild-type and 11 cysteine substituted mutants. The drug concentrations are each 2 × EC50 in tadpole loss of righting reflexes assays and similarly modulate wild-type receptor currents: 3.2 μM etomidate (*red*); 5 μM propofol (*white*); and 2.5 μM alphaxalone (*purple*). Of note, EC5 GABA concentrations were established in comparison with maximal GABA responses. Thus, in mutants where maximal GABA efficacy is low (table 2), enhancements ratios greater than 20 are possible. Statistically significant differences from wild-type results are indicated by $^*P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$.

of functional receptor states in both control modification and protection experiments.[6,21] Initial protection conditions included 10 μM etomidate, 20 μM propofol, or 10 μM ALX along with GABA and pCMBS. In some mutant receptors that displayed low apparent affinity for anesthetics, we also used fivefold higher protecting anesthetic concentrations. In these cases, we used equivalent high concentrations of at least one other anesthetic to test for drug-specific protection.

Normalized modification data and rate analyses for nine mutations are shown in figure 4 and summarized in figure 4J.[21] The apparent rate of modification of α1β3T262Cγ2L receptors (fig. 4A) was unaffected by etomidate (*red symbols* and *lines*), but accelerated by propofol (*green symbols* and *lines*). Modification of α1β3T266Cγ2L receptors (fig. 4B) was accelerated by all three anesthetics, suggesting an allosteric effect. β3M286C protection was fully consistent with previous SCAMP studies of α1β2M286Cγ2L receptors, showing that both etomidate and propofol block modification, while ALX weakly accelerates pCMBS modification (summarized in fig. 4J).[20,21] Modification of α1β3G287Cγ2L receptors (fig. 4C) was unaffected by the three anesthetics. Modification of α1β3F289Cγ2L receptors was weakly blocked by 10 μM etomidate, unaffected by 20 μM propofol, and accelerated by 10 μM ALX (data not shown). Because this mutant was insensitive to anesthetics (fig. 2), we also tested 50 μM etomidate, which inhibited the apparent rate of β3F289C modification over tenfold, while neither 100 μM propofol nor 50 μM ALX inhibited modification (fig. 4D). Modification of α1β3V290Cγ2L receptors (fig. 4E) was strongly blocked by 10 μM etomidate, 20 μM propofol, and 10 μM ALX. To test whether β3V290C modification was allosterically inhibited by anesthetics that do not bind in β^+–α^- sites, we also tested the effect of 10 μM mTFD-MPAB, a potent barbiturate that selectively binds to $GABA_A$ receptor α^+–β^- and γ^+–β^- transmembrane interfaces.[8] Modification of receptors with β3V290C mutations was unaffected by 8 μM mTFD-MPAB (fig. 4J), indicating that inhibition of modification by etomidate, propofol, and ALX was likely steric rather than allosteric.

Modification of α1β3F293Cγ2L receptors was accelerated by etomidate and propofol, but unaffected by 10 μM ALX. Increasing ALX to 20 μM (fig. 4F, *dashed purple lines*) or 50 μM (fig. 4F, *solid purple lines*) resulted in significantly reduced rates of β3F293C modification in comparison to 50 μM etomidate and 100 μM propofol (fig. 4F). Modification of α1β3L297Cγ2L receptors was unaffected by low concentrations of etomidate, propofol, or ALX (not shown). Because α1β3L297Cγ2L is relatively insensitive to ALX (fig. 2), we performed additional SCAMP experiments with 50 μM ALX *versus* 50 μM etomidate in this mutant,

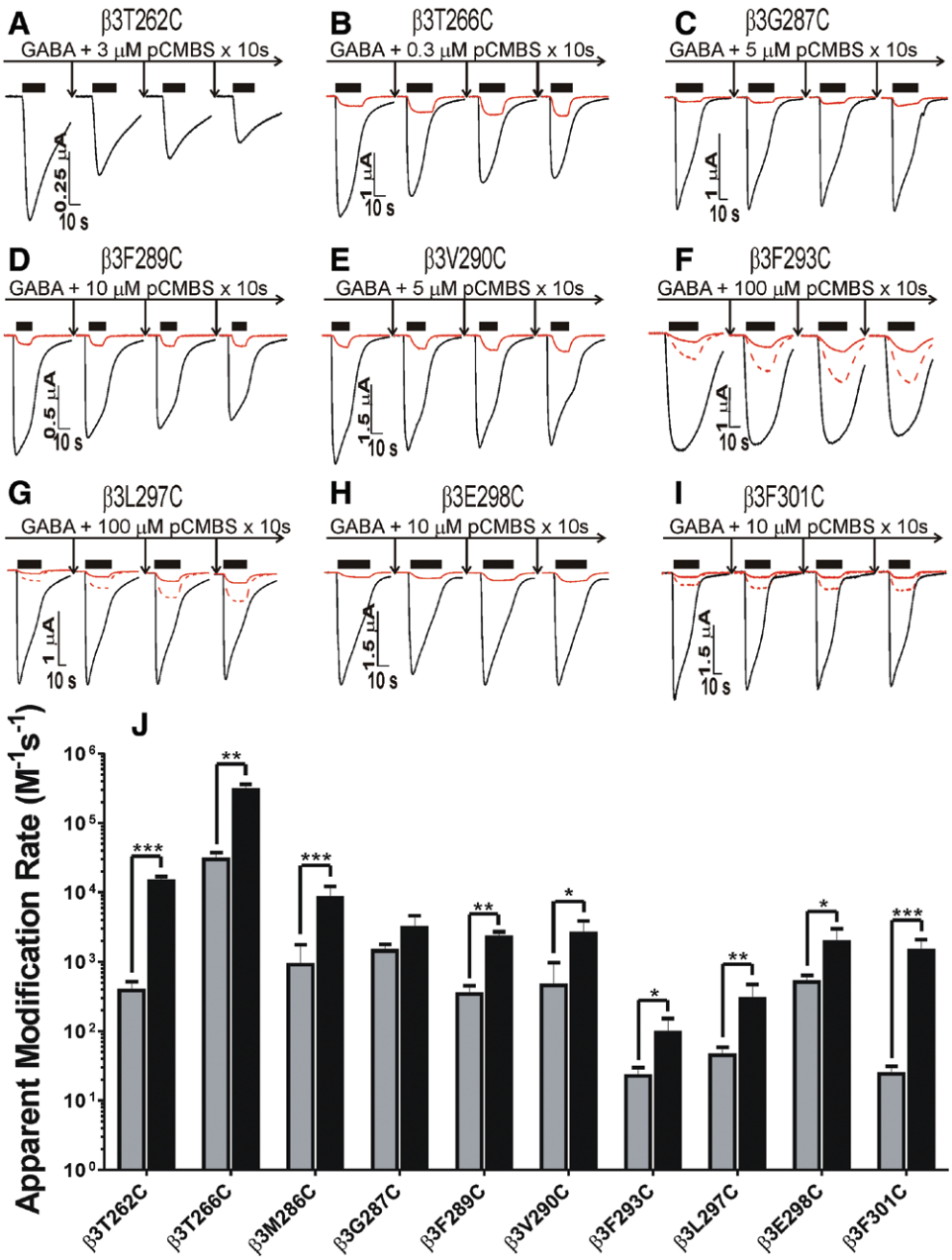

Fig. 3. Effects of para-chloromercuribenzenesulfonic acid sodium salt (pCMBS) exposure on cysteine-substituted γ-aminobutyric acid type A ($GABA_A$) receptors in the absence and presence of γ-aminobutyric acid (GABA). (*A–I*) are each labeled with the relevant cysteine mutant and show current traces from an oocyte stimulated with either EC5 GABA (*red*) or maximal GABA (*black*) before and after three cycles of pCMBS + GABA exposure and ND96 wash. (*A*) omits EC5 traces, which diminished in parallel with high GABA responses. EC5 traces in (*F), (G)*, and (*I*) are duplicated at 3 × magnitude (*red dashed lines*) to better illustrate the effects of pCMBS modification. Specific modification conditions are indicated in each panel. GABA exposure periods are indicated by *black bars over traces*. (*J*) summarizes the apparent rates of receptor modification (average ± SEM) in the absence (*gray bars*) and presence of GABA (*black bars*). Corresponding examples of rate analyses are shown in figures 4A–I. With the exception of β3G287C, GABA significantly accelerated the apparent modification rates. **P* < 0.05, ***P* < 0.01, or ****P* < 0.001.

revealing inhibition by ALX, but not etomidate (fig. 4G). Modification of α1β3E298Cγ2L receptors (fig. 4H) was unaffected by any of the anesthetics. Modification of α1β3F301Cγ2L receptors (fig. 4I) was weakly, but significantly, blocked by 10 to 20 μM ALX and unaffected by 10 to 20 μM etomidate.

On the opposite face of the transmembrane β^+–α^- cleft, Hosie *et al.*[13] identified mutant effects on neurosteroid sensitivity at three residues in α1-M1: α1T237, α1I239, and α1Q242 (table 1). We previously reported that receptors with both α1I239C and α1Q242C mutations are unaffected by pCMBS, precluding SCAMP studies.[6] To supplement

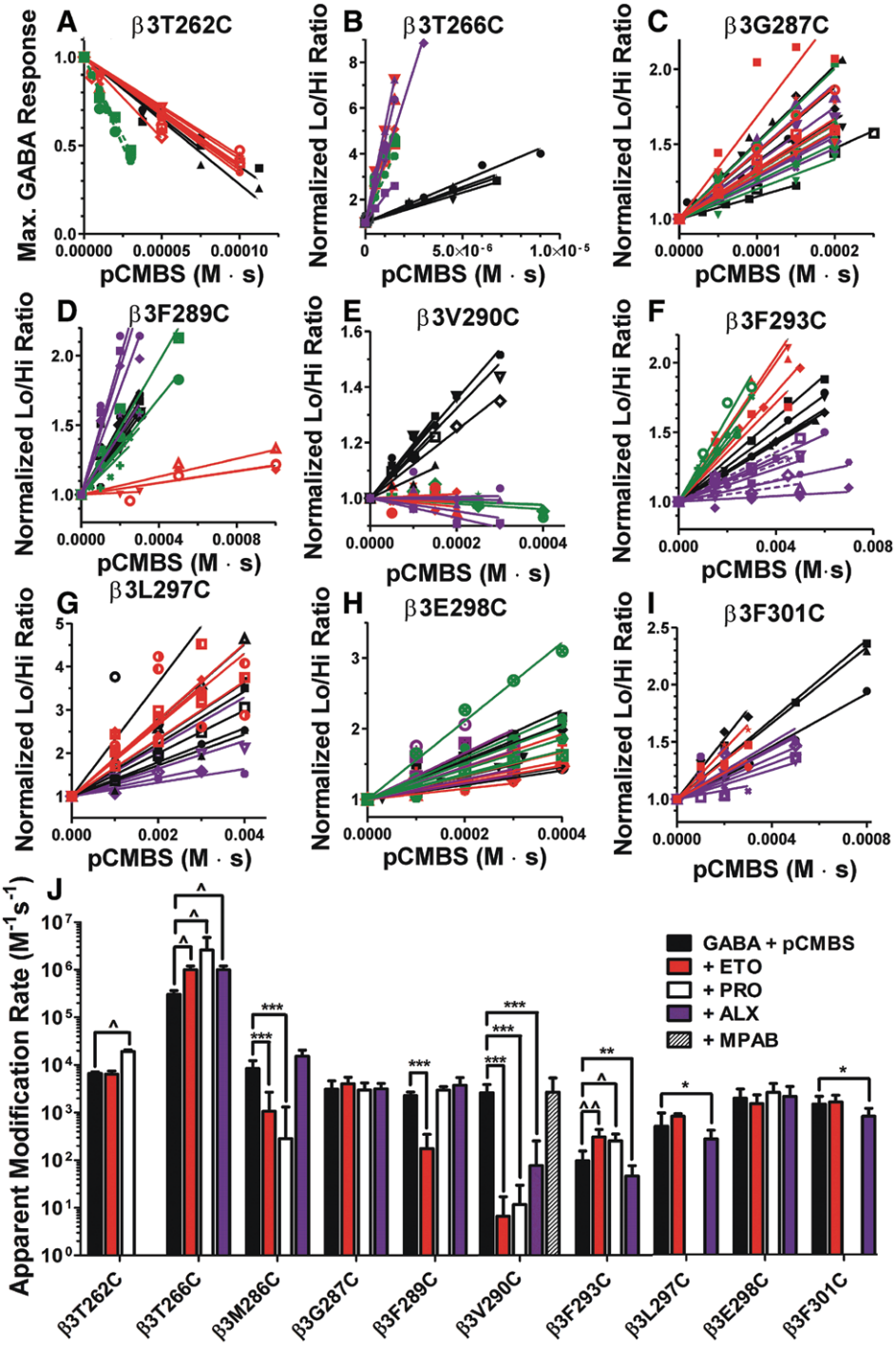

Fig. 4. Anesthetic protection of substituted cysteine mutant γ-aminobutyric acid type A ($GABA_A$) receptors. (*A–I*), labeled by mutation, show individual oocyte data and linear fits for control modification (GABA + para-chloromercuribenzenesulfonic acid sodium salt [pCMBS]; *black symbols and lines*), and modification in the presence of etomidate (*red symbols and lines*), propofol (*green symbols and lines*), and alphaxalone (*purple symbols and lines*) results. Corresponding example current traces for control modification are shown in figure 3. Anesthetic concentrations were 10 μM etomidate, 20 μM propofol, and 10 μM alphaxalone, except for β3F289C, β3F293C, and β3L297C, where fivefold higher concentrations were used. Data for β3M286C are not shown because we have previously reported similar results.[21] (*J*) summarizes mean ± SD rates (fitted linear slopes) for all ten cysteine-substituted mutants on a logarithmic scale. Results for 8 μM mTFD-MPAB effects on β3V290C modification (N = 6) are included. Negative slopes for β3T262C and β3V290C were inverted for rate comparisons. Two-way ANOVA analysis was used to assess whether addition of anesthetics significantly altered the apparent rates of modification relative to control conditions with GABA + pCMBS in each mutant. Protection is inferred in cases where addition of anesthetics significantly reduced modification rates. *Significantly reduced modification rate, $P < 0.05$; **Significantly reduced modification rate, $P < 0.01$; ***Significantly reduced modification rate, $P < 0.001$; ^Significantly increased modification rate, $P < 0.05$; ^^Significantly increased modification rate, $P < 0.01$.

our studies of β3-M2 and β3-M3 residues, we used SCAMP to test whether ALX protects the cysteine substitution at α1T237. No inhibition of pCMBS modification rates in α1T237Cβ3γ2L receptors by 10 μM ALX was observed (data not shown), whereas 10 μM etomidate inhibited modification, in agreement with previous results.[6]

Discussion

Major Findings

Our aims in this study were to assess hypothesized ALX contacts with β3 sidechains that face transmembrane β^+–α^- clefts in α1β3γ2L $GABA_A$ receptors, and to compare these with etomidate and propofol contacts. Using electrophysiology, we studied ten mutant receptors with single cysteine-substitutions in β3-M2 or β3-M3 helices, in which the sulfhydryl modifier pCMBS produced irreversible functional changes. Based on drug-specific inhibition of pCMBS modification, we infer a number of anesthetic contact residues: etomidate binds near β3M286, β3F289, and β3V290 (fig. 5A); propofol binds near β3M286 and β3V290 (fig. 5B); and ALX binds near β3V290, β3F293, β3L297, and β3F301 (fig. 5C). Mapping these residues onto our α1β3γ2L structural model (figs. 5D–I) suggests that all three anesthetics bind in transmembrane β^+–α^- intersubunit clefts, with overlapping etomidate and propofol sites extending from the middle of β3-M3 (near β3V290) extracellularly (figs. 5, D and E), and the ALX site extending from β3V290 intracellularly (fig. 5F).

Alphaxalone and Neurosteroids Bind to Inner Transmembrane β^+–α^- Sites. Single-point mutations that affect neurosteroid sensitivity in heteromeric mammalian $GABA_A$ receptors (table 1) are found throughout the transmembrane β^+–α^- cleft. Our SCAMP results for ALX provide evidence of contact with four inner β3-M3 residues facing the β^+–α^- interface. The strongest prior evidence for an inner transmembrane β^+–α^- neurosteroid site is β3F301 photolabeling with 6-AziP,[22] but the use of homomeric β3 receptors and failure to test if neurosteroids block 6-AziP labeling make it far weaker than studies in heteromeric receptors using photolabeling derivatives of etomidate and propofol.[26] Mutations at both α1I239 and α1Q242, located opposite β3F293 in our structural model (fig. 1), impair receptor sensitivity to neurosteroids[13,14,28] and α1Q242C confers insensitivity to ALX, but not to etomidate (unpublished data). The lack of pCMBS-induced effects in receptors with α1I239C and α1Q242C mutations[6] precludes SCAMP tests and contrasts with our current findings in inner β3-M3 mutants. Other indirect support for inner transmembrane neurosteroid sites include evidence that a membrane-impermeant steroid positively modulates $GABA_A$ receptors only when applied intracellularly.[23] Docking calculations using the β3 homomeric $GABA_A$ receptor structure[30] also locate pregnanolone and allopregnanolone sites near both β3F301 and β3L297.[39]

Previous functional, SCAMP, and photolabeling evidence (table 1) all locate etomidate and propofol sites in outer transmembrane β^+–α^- clefts. In comparing ALX contacts in β3-M3 with those for etomidate and propofol, we found that, with the exception of β3V290C, ALX contacts were mutually exclusive with propofol or etomidate contacts. We also recently reported that etomidate contacts α1L232, and that both etomidate and propofol contact α1M236, while ALX contacts neither.[5] Altogether, our current results indicate that ALX binds in inner transmembrane β^+–α^- cleft sites abutting outer transmembrane etomidate/propofol sites, with possible contact of outer and inner sites near β3V290.

Neurosteroids enhance $GABA_A$ receptor photolabeling by etomidate derivatives[28] and neurosteroid-etomidate combinations synergize in both enhancing $GABA_A$ receptor gating and anesthetizing animals.[29] An allosteric mechanism for this synergy through mutual coupling of sites to channel gating is suggested by our observations that both etomidate and propofol accelerate pCMBS modification of β3F293C in the ALX sites, while ALX accelerates pCMBS modification at β3M286C in the etomidate/propofol sites. Direct contact between neurosteroids and etomidate in abutting sites could also mutually enhance drug binding, contributing to functional synergy.

Propofol and Etomidate Bind to Outer Transmembrane β^+–α^- Sites. Our current results extend the map of propofol and etomidate contacts on the β+ aspect of the outer β^+–α^- sites (table 1; fig. 5). Functional and SCAMP results with β3M286C echoed previous studies of β2M286C.[20,21] We identified two additional etomidate contact residues, β3F289 and β3V290, while propofol protects β3V290C but not β3F289C. Thus, the β^+–α^- sites for propofol and etomidate overlap, agreeing with previous SCAMP and photolabel competition results (table 1).[5,8,12] Interestingly, despite evidence that propofol and etomidate might contact β2/3N265 on the M2 helix (table 1), we found no evidence of contact at β3T262 or β3T266 that also abut β^+–α^- interfaces in structural models (fig. 1).

Mutant Functional Effects Reflect Allosteric Linkages, Not Drug-receptor Contacts. The functional effects of both cysteine-substitution and pCMBS modification provide insight into allosteric linkages and aqueous accessibility at the residues we studied. Spanning from M286 to E298, most β3-M3 cysteine mutations altered GABA EC_{50} and/or GABA efficacy (table 2), indicating that this region is coupled to ion channel gating. Similar observations were made in a series of α1-M1 cysteine substitutions.[6] Cysteine mutants throughout β3-M3 were also accessible to pCMBS, indicating an aqueous pathway extending intracellularly to at least β3F301, and echoing similar findings on the β1-M2 helix.[40]

Mutant functional analyses underlie many of the hypotheses we have tested (table 1) and it is tempting to infer drug contacts from the altered anesthetic sensitivities of cysteine mutants (fig. 2). However, we recently compared SCAMP with tryptophan mutant drug sensitivity for two photolabeled residues and four anesthetics, finding perfect agreement between SCAMP and photolabeling, but poor concordance with

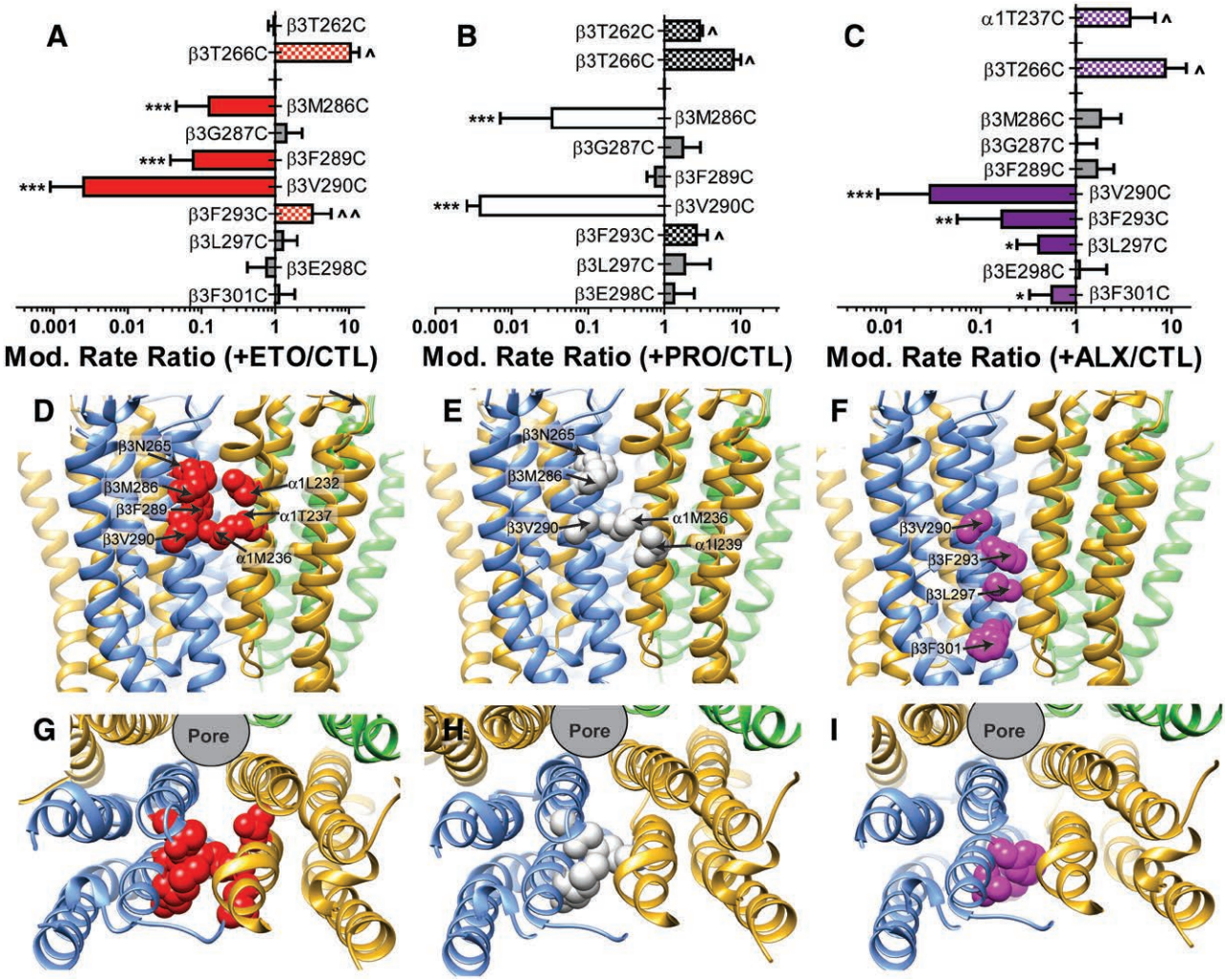

Fig. 5. Summary of substituted cysteine modification and protection results by anesthetic drug. Panels (*A–C*) summarize the ratio of modification rates (mean ± SD) in the presence *versus* absence of anesthetic for each drug at the mutations used in the current study. Cases where no significant change was observed are indicated by *gray bars*. Cases where anesthetics caused significant slowing of modification are identified by *solid colored bars* and those where anesthetics produced significant acceleration of modification are identified by *checked bars* with the same color scheme (etomidate [ETO], *red*; propofol [PRO], *white*; alphaxalone [ALX], *purple*). Contact between anesthetics and sidechains is inferred in cases where modification is inhibited. Panels (*D–F*) depict transmembrane domain backbone ribbon structure of our α1β3γ2L homology model as viewed from the side. Contact residues, based on both photolabeling and substituted cysteine modification-protection studies, are identified for each drug in separate panels as colored and labeled space-filling models. Panels (*G–I*) depict the same models and contact sidechains viewed from the extracellular space, with the extracellular domains removed. Subunit color coding: α1 = yellow; β3 = blue; γ2L = green. *Significantly reduced modification rate, $P < 0.05$; **Significantly reduced modification rate, $P < 0.01$; ***Significantly reduced modification rate, $P < 0.001$; ^Significantly increased modification rate, $P < 0.05$; ^^Significantly increased modification rate, $P < 0.01$.

mutant drug sensitivities.[5] There are multiple other examples of SCAMP identifying anesthetic contacts in $GABA_A$ receptors that weren't photolabeled,[5,6,26,41] but only one published report of SCAMP disagreeing with photolabeling.[25]

SCAMP Conditionally Reflects Drug-receptor Contacts. Our SCAMP approach requires functional heterologous receptor expression, quantifiably consistent cysteine modification effects, and drug occupation of a large fraction of sites.[26] Even under these conditions, we cannot formally rule out allosteric effects in SCAMP experiments. However, allosteric mechanisms should strongly link the functional effects of different anesthetics to inhibition of modification in relevant mutants. Comparing figures 2 and 4J, such correlations are absent at many positions where modification was inhibited: F289, V290, F293, and F301. Moreover, drug specificity was demonstrable at every protected cysteine (fig. 4J). Thus, our SCAMP results are more compatible with a steric mechanism rather than an allosteric mechanism for inhibiting pCMBS modification. Inferences of steric interactions between receptor-bound drugs and substituted cysteines are strengthened when protection is concentration-dependent and profound. ALX protection at β3F293C, β3L297C, and

β3F301C was relatively weak compared to results for etomidate, propofol, and mTFD-MPAB at some of their outer transmembrane contacts.[5,6,21] For β3F293C and β3L297C, this is attributable to low ALX affinity (see next paragraph). The β3F301C sidechain may be located at the periphery of the steroid site, limiting ALX protection at this position.

In three mutant receptors, α1β3F289Cγ2L, α1β3F293Cγ2L, and α1β3L297Cγ2L, high anesthetic concentrations demonstrated concentration-dependent block of pCMBS modification. In these mutants, weak EC5 enhancement (fig. 2) indicated weak drug binding based on the Monod-Wyman-Changeux allosteric principle that positive gating modulation reflects the relative affinity of ligands for active (open) *versus* inactive (closed) receptors. Thus, weak EC5 enhancement relative to wild-type implies reduced drug affinity for GABA-activated receptors and a need for high drug concentrations to occupy most binding sites. In addition, α1β3F293Cγ2L receptors were characterized by low GABA efficacy, with maximal GABA activating only about 16% of these receptors (table 2) under control modification conditions. With addition of etomidate or propofol, the fraction of activated and desensitized receptors increased, allosterically accelerating β3F293C modification (fig. 4J). Adding ALX to high GABA likely produced two opposing effects on α1β3F293Cγ2L modification: increased activation/desensitization that accelerates modification, and steric protection that inhibits modification. In initial experiments, 10 μM ALX produced approximate balance in these opposing effects, while higher ALX concentrations resulted in overall slowing of modification.

Intrasubunit Pockets. Crystallographic studies of pentameric ligand-gated ion channels reveal that small anesthetics and alcohols can occupy both intersubunit and intrasubunit transmembrane pockets.[42–44] In this study, we examined two mutations (β3G287C and β3E298C) that are predicted to face the β3 intrasubunit helix bundle pocket, in both outer and inner regions of β3-M3 (fig. 1). While we observed altered GABA sensitivity as evidence of pCMBS access and modification in these mutants, no anesthetic protection was observed (figs. 4 and 5). These results are evidence against the presence of positively modulating anesthetic sites in β3 intrasubunit pockets.

Conclusions and Significance

Endogenous and synthetic neurosteroids are potent neuromodulators with broad therapeutic potential. Our current SCAMP studies locate positively modulating ALX sites on α1β3γ2L $GABA_A$ receptors in inner transmembrane β^+–α^- intersubunit clefts. These neurosteroid sites are adjacent to outer transmembrane β^+–α^- sites where etomidate and propofol act, suggesting both direct and indirect mechanisms for cooperativity between neurosteroids and etomidate.[28,29] Two other outer transmembrane intersubunit sites, in α^+–β^- and γ^+–β^- clefts, bind propofol and barbiturates.[5,8] No ligands have yet been identified for the transmembrane α^+–γ^- cleft and the inner transmembrane portions of α^+–β^- and γ^+–β^- interfaces, but membrane lipids probably modulate ion channel activity by interacting with transmembrane intersubunit clefts.[45] In summary, large portions of the five transmembrane intersubunit clefts in α1β3γ2L $GABA_A$ receptors are allosterically coupled to ion channel gating. Subregions of these clefts form sites for hydrophobic modulators that in several cases, including that of neurosteroids, display remarkable drug selectivity. Structural variations in these intersubunit interfaces also contribute to subtype-selective $GABA_A$ receptor pharmacology.

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Competing Interests

The authors declare no competing interests.

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References

1. Porcu P, Barron AM, Frye CA, Walf AA, Yang SY, He XY, Morrow AL, Panzica GC, Melcangi RC: Neurosteroidogenesis today: Novel targets for neuroactive steroid synthesis and action and their relevance for translational research. J Neuroendocrinol 2016; 28:12351
2. Olsen RW, Sieghart W: $GABA_A$ receptors: Subtypes provide diversity of function and pharmacology. Neuropharmacology 2009; 56:141–8
3. Sigel E, Steinmann ME: Structure, function, and modulation of $GABA_A$ receptors. J Biol Chem 2012; 287:40224–31
4. Baumann SW, Baur R, Sigel E: Forced subunit assembly in α1β2γ2 $GABA_A$ receptors. Insight into the absolute arrangement. J Biol Chem 2002; 277:46020–5
5. Nourmahnad A, Stern AT, Hotta M, Stewart DS, Ziemba AM, Szabo A, Forman SA: Tryptophan and cysteine mutations in

M1 helices of α1β3γ2L γ-aminobutyric acid type A receptors indicate distinct intersubunit sites for four intravenous anesthetics and one orphan site. Anesthesiology 2016; 125:1144–58

6. Stewart DS, Hotta M, Li GD, Desai R, Chiara DC, Olsen RW, Forman SA: Cysteine substitutions define etomidate binding and gating linkages in the α-M1 domain of γ-aminobutyric acid type A ($GABA_A$) receptors. J Biol Chem 2013; 288:30373–86
7. Stewart D, Desai R, Cheng Q, Liu A, Forman SA: Tryptophan mutations at azi-etomidate photo-incorporation sites on α1 or β2 subunits enhance $GABA_A$ receptor gating and reduce etomidate modulation. Mol Pharmacol 2008; 74:1687–95
8. Chiara DC, Jayakar SS, Zhou X, Zhang X, Savechenkov PY, Bruzik KS, Miller KW, Cohen JB: Specificity of intersubunit general anesthetic-binding sites in the transmembrane domain of the human α1β3γ2 γ-aminobutyric acid type A ($GABA_A$) receptor. J Biol Chem 2013; 288:19343–57
9. Stewart DS, Pierce DW, Hotta M, Stern AT, Forman SA: Mutations at β N265 in γ-aminobutyric acid type A receptors alter both binding affinity and efficacy of potent anesthetics. PLoS One 2014; 9:e111470
10. Li GD, Chiara DC, Sawyer GW, Husain SS, Olsen RW, Cohen JB: Identification of a $GABA_A$ receptor anesthetic binding site at subunit interfaces by photolabeling with an etomidate analog. J Neurosci 2006; 26:11599–605
11. Chiara DC, Dostalova Z, Jayakar SS, Zhou X, Miller KW, Cohen JB: Mapping general anesthetic binding site(s) in human α1β3 γ-aminobutyric acid type A receptors with [^{3}H]TDBzl-etomidate, a photoreactive etomidate analogue. Biochemistry 2012; 51:836–47
12. Jayakar SS, Zhou X, Chiara DC, Dostalova Z, Savechenkov PY, Bruzik KS, Dailey WP, Miller KW, Eckenhoff RG, Cohen JB: Multiple propofol-binding sites in a γ-aminobutyric acid type A receptor (GABAAR) identified using a photoreactive propofol analog. J Biol Chem 2014; 289:27456–68
13. Hosie AM, Wilkins ME, da Silva HM, Smart TG: Endogenous neurosteroids regulate $GABA_A$ receptors through two discrete transmembrane sites. Nature 2006; 444:486–9
14. Akk G, Li P, Bracamontes J, Reichert DE, Covey DF, Steinbach JH: Mutations of the GABA-A receptor α1 subunit M1 domain reveal unexpected complexity for modulation by neuroactive steroids. Mol Pharmacol 2008; 74:614–27
15. Belelli D, Lambert JJ, Peters JA, Wafford K, Whiting PJ: The interaction of the general anesthetic etomidate with the γ-aminobutyric acid type A receptor is influenced by a single amino acid. Proc Natl Acad Sci USA 1997; 94:11031–6
16. Desai R, Ruesch D, Forman SA: γ-Amino butyric acid type A receptor mutations at β2N265 alter etomidate efficacy while preserving basal and agonist-dependent activity. Anesthesiology 2009; 111:774–84
17. Maldifassi MC, Baur R, Sigel E: Functional sites involved in modulation of the $GABA_A$ receptor channel by the intravenous anesthetics propofol, etomidate and pentobarbital. Neuropharmacology 2016; 105:207–14
18. Siegwart R, Krähenbühl K, Lambert S, Rudolph U: Mutational analysis of molecular requirements for the actions of general anaesthetics at the γ-aminobutyric acid A receptor subtype, α1β2γ2. BMC Pharmacol 2003; 3:13
19. Krasowski MD, Nishikawa K, Nikolaeva N, Lin A, Harrison NL: Methionine 286 in transmembrane domain 3 of the $GABA_A$ receptor beta subunit controls a binding cavity for propofol and other alkylphenol general anesthetics. Neuropharmacology 2001; 41:952–64
20. Bali M, Akabas MH: Defining the propofol binding site location on the $GABA_A$ receptor. Mol Pharmacol 2004; 65:68–76
21. Stewart DS, Hotta M, Desai R, Forman SA: State-dependent etomidate occupancy of its allosteric agonist sites measured in a cysteine-substituted $GABA_A$ receptor. Mol Pharmacol 2013; 83:1200–8
22. Chen ZW, Manion B, Townsend RR, Reichert DE, Covey DF, Steinbach JH, Sieghart W, Fuchs K, Evers AS: Neurosteroid analog photolabeling of a site in the third transmembrane domain of the β3 subunit of the $GABA_A$ receptor. Mol Pharmacol 2012; 82:408–19
23. Akk G, Shu HJ, Wang C, Steinbach JH, Zorumski CF, Covey DF, Mennerick S: Neurosteroid access to the $GABA_A$ receptor. J Neurosci 2005; 25:11605–13
24. Hibbs RE, Gouaux E: Principles of activation and permeation in an anion-selective Cys-loop receptor. Nature 2011; 474:54–60
25. Stern AT, Forman SA: A cysteine substitution probes β3H267 interactions with propofol and other potent anesthetics in α1β3γ2L γ-aminobutyric acid type A receptors. Anesthesiology 2016; 124:89–100
26. Forman SA, Miller KW: Mapping general anesthetic sites in heteromeric γ-aminobutyric acid type A receptors reveals a potential for targeting receptor subtypes. Anesth Analg 2016; 123:1263–73
27. Westergard T, Salari R, Martin JV, Brannigan G: Interactions of L-3,5,3'-triiodothyronine [corrected], allopregnanolone, and ivermectin with the $GABA_A$ receptor: Evidence for overlapping intersubunit binding modes. PLoS One 2015; 10:e0139072
28. Li GD, Chiara DC, Cohen JB, Olsen RW: Neurosteroids allosterically modulate binding of the anesthetic etomidate to γ-aminobutyric acid type A receptors. J Biol Chem 2009; 284:11771–5
29. Li P, Bracamontes JR, Manion BD, Mennerick S, Steinbach JH, Evers AS, Akk G: The neurosteroid 5β-pregnan-3α-ol-20-one enhances actions of etomidate as a positive allosteric modulator of α1β2γ2L $GABA_A$ receptors. Br J Pharmacol 2014; 171:5446–57
30. Miller PS, Aricescu AR: Crystal structure of a human $GABA_A$ receptor. Nature 2014; 512:270–5
31. Savechenkov PY, Zhang X, Chiara DC, Stewart DS, Ge R, Zhou X, Raines DE, Cohen JB, Forman SA, Miller KW, Bruzik KS: Allyl m-trifluoromethyldiazirine mephobarbital: An unusually potent enantioselective and photoreactive barbiturate general anesthetic. J Med Chem 2012; 55:6554–65
32. Bandyopadhyaya AK, Manion BD, Benz A, Taylor A, Rath NP, Evers AS, Zorumski CF, Mennerick S, Covey DF: Neurosteroid analogues. 15. A comparative study of the anesthetic and GABAergic actions of alphaxalone, Δ16-alphaxalone and their corresponding 17-carbonitrile analogues. Bioorg Med Chem Lett 2010; 20:6680–4
33. Husain SS, Stewart D, Desai R, Hamouda AK, Li SG, Kelly E, Dostalova Z, Zhou X, Cotten JF, Raines DE, Olsen RW, Cohen JB, Forman SA, Miller KW: p-Trifluoromethyldiazirinyl-etomidate: A potent photoreactive general anesthetic derivative of etomidate that is selective for ligand-gated cationic ion channels. J Med Chem 2010; 53:6432–44
34. Tonner PH, Poppers DM, Miller KW: The general anesthetic potency of propofol and its dependence on hydrostatic pressure. Anesthesiology 1992; 77:926–31
35. Borghese CM, Hicks JA, Lapid DJ, Trudell JR, Harris RA: $GABA_A$ receptor transmembrane amino acids are critical for alcohol action: Disulfide cross-linking and alkyl methanethiosulfonate labeling reveal relative location of binding sites. J Neurochem 2014; 128:363–75
36. Chang Y, Weiss DS: Allosteric activation mechanism of the α1β2γ2 gamma-aminobutyric acid type A receptor revealed by mutation of the conserved M2 leucine. Biophys J 1999; 77:2542–51
37. Bali M, Akabas MH: Gating-induced conformational rearrangement of the γ-aminobutyric acid type A receptor β-α subunit interface in the membrane-spanning domain. J Biol Chem 2012; 287:27762–70
38. Bali M, Jansen M, Akabas MH: GABA-induced intersubunit conformational movement in the $GABA_A$ receptor

α1M1-β2M3 transmembrane subunit interface: Experimental basis for homology modeling of an intravenous anesthetic binding site. J Neurosci 2009; 29:3083–92

39. Alvarez LD, Estrin DA: Exploring the molecular basis of neurosteroid binding to the β3 homopentameric $GABA_A$ receptor. J Steroid Biochem Mol Biol 2015; 154:159–67
40. Goren EN, Reeves DC, Akabas MH: Loose protein packing around the extracellular half of the $GABA_A$ receptor β1 subunit M2 channel-lining segment. J Biol Chem 2004; 279:11198–205
41. Woll KA, Dailey WP, Brannigan G, Eckenhoff RG: Shedding light on anesthetic mechanisms: Application of photoaffinity ligands. Anesth Analg 2016; 123:1253–62
42. Nury H, Van Renterghem C, Weng Y, Tran A, Baaden M, Dufresne V, Changeux JP, Sonner JM, Delarue M, Corringer PJ: X-ray structures of general anaesthetics bound to a pentameric ligand-gated ion channel. Nature 2011; 469:428–31
43. Spurny R, Billen B, Howard RJ, Brams M, Debaveye S, Price KL, Weston DA, Strelkov SV, Tytgat J, Bertrand S, Bertrand D, Lummis SC, Ulens C: Multisite binding of a general anesthetic to the prokaryotic pentameric Erwinia chrysanthemi ligand-gated ion channel (ELIC). J Biol Chem 2013; 288:8355–64
44. Sauguet L, Howard RJ, Malherbe L, Lee US, Corringer PJ, Harris RA, Delarue M: Structural basis for potentiation by alcohols and anaesthetics in a ligand-gated ion channel. Nat Commun 2013; 4:1697
45. Althoff T, Hibbs RE, Banerjee S, Gouaux E: X-ray structures of GluCl in apo states reveal a gating mechanism of Cys-loop receptors. Nature 2014; 512:333–7

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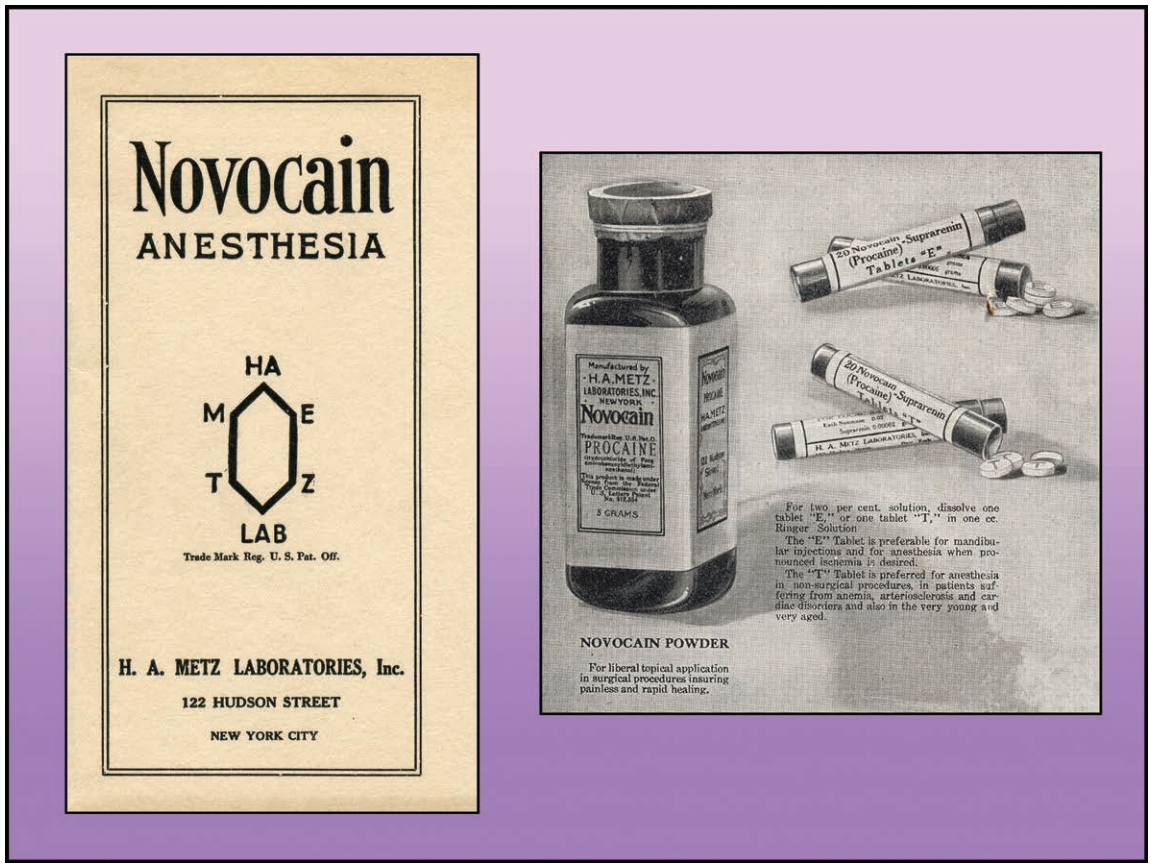
Lord of the Ring: General Metz and Local Anesthesia



Years after trademarking procaine as "Novocain," U.S.-born German-American Herman A. Metz discovered that this action had unintentionally complicated the American military's procurement of World War I local anesthetics. A colonel and eventually a general in the National Guard, Metz trademarked a hexagonal ring logo bearing letters from his company, HA METZ LAB. Metz, his wife, and founding father Alexander Hamilton were memorialized by the naming of Hamilton-Metz Field in Brooklyn, New York. (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

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