Two Etomidate Sites in $\alpha1\beta2\gamma2$ γ-Aminobutyric Acid Type A Receptors Contribute Equally and Noncooperatively to Modulation of Channel Gating

Grigori Guitchounts, B.A.,* Deirdre S. Stewart, Ph.D.,† Stuart A. Forman, M.D., Ph.D.‡

ABSTRACT

Background: Etomidate is a potent hypnotic agent that acts *via* γ-aminobutyric acid receptor type A ($GABA_A$) receptors. Evidence supports the presence of two etomidate sites per $GABA_A$ receptor, and current models assume that each site contributes equally and noncooperatively to drug effects. These assumptions remain untested.

Methods: We used concatenated dimer ($\beta2$-$\alpha1$) and trimer ($\gamma2$-$\beta2$-$\alpha1$) $GABA_A$ subunit assemblies that form functional $\alpha1\beta2\gamma2$ channels, and inserted $\alpha1$M236W etomidate site mutations into both dimers ($\beta2$-$\alpha1$M236W) and trimers ($\gamma2$-$\beta2$-$\alpha1$M236W). Wild-type or mutant dimers (D^{wt} or $D^{\alpha M236W}$) and trimers (T^{wt} or $T^{\alpha M236W}$) were coexpressed in *Xenopus* oocytes to produce four types of channels: $D^{wt}T^{wt}$, $D^{\alpha M236W}T^{wt}$, $D^{wt}T^{\alpha M236W}$, and $D^{\alpha M236W}T^{\alpha M236W}$. For each channel type, two-electrode voltage clamp was performed to quantitatively assess GABA EC_{50}, etomidate modulation (left shift), etomidate direct activation, and other functional parameters affected by αM236W mutations.

Results: Concatenated wild-type $D^{wt}T^{wt}$ channels displayed etomidate modulation and direct activation similar to $\alpha1\beta2\gamma2$ receptors formed with free subunits. $D^{\alpha M236W}T^{\alpha M236W}$ receptors also displayed altered GABA sensitivity and etomidate modulation similar to mutated channels formed with free subunits. Both single-site mutant receptors ($D^{\alpha M236W}T^{wt}$ and $D^{wt}T^{\alpha M236W}$) displayed indistinguishable functional properties and equal gating energy changes for GABA activation (-4.9 ± 0.48 *vs.* -4.7 ± 0.48 kJ/mol, respectively) and etomidate modulation (-3.4 ± 0.49 *vs.* -3.7 ± 0.38 kJ/mol, respectively), which together accounted for the differences between $D^{wt}T^{wt}$ and $D^{\alpha M236W}T^{\alpha M236W}$ channels.

Conclusions: These results support the hypothesis that the two etomidate sites on $\alpha1\beta2\gamma2$ $GABA_A$ receptors contribute equally and noncooperatively to drug interactions and gating effects.

What We Already Know about This Topic

- Etomidate binds to two sites on the γ-aminobutyric acid receptor type A ($GABA_A$) receptor, but the contribution of each site to etomidate effects remains untested

What This Article Tells Us That Is New

- Etomidate interactions at each of its $GABA_A$ receptor sites produce equal and additive gating modulation effects

IN central nervous system neurons, γ-aminobutyric acid type A ($GABA_A$) receptors are inhibitory receptors, and major targets of general anesthetics such as etomidate and propofol. $GABA_A$ receptors contain five homologous subunits arranged around a chloride-conducting pore. Each subunit includes a large extracellular domain and four transmembrane domains (M1–M4).[1] The isoform containing two $\alpha1$, two $\beta2$, and one $\gamma2$ is the most common, and displays physiology and pharmacology similar to synaptic receptors.[2]

Etomidate is a potent sedative/hypnotic drug that acts by potentiating $GABA_A$ receptor activity.[3–5] Etomidate enhances $\alpha1\beta2\gamma2$ receptor currents elicited by low GABA concentrations, whereas high concentrations of etomidate directly activate these channels.[6,7] Structural and pharmacological studies show that both of these effects are mediated by a single class of etomidate binding sites,[8] and quantitative model analysis suggests that two equivalent etomidate sites are present on each $GABA_A$ receptor.[7] A photo-activatable etomidate analog, [^{3}H]azi-etomidate, labels affinity-purified bovine brain $GABA_A$ receptors at both αM236 (located in the M1 domain) and βM286 (located in the M3 domain).[9] Etomidate inhibits photolabeling at αM236 and βM286 in parallel, suggesting that both residues abut a common etomidate site. Homology modeling based on

* Research Technologist, Department of Neurobiology, Boston University, Boston, Massachusetts. † Research Associate, ‡ Associate Professor, Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Boston, Massachusetts.

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Address correspondence to Dr. Forman: Department of Anesthesia, Critical Care and Pain Medicine, Jackson 444, Massachusetts General Hospital, 55 Fruit Street, Boston, Massachusetts 02114. saforman@partners.org. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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the *Torpedo* nicotinic acetylcholine receptor structure suggests that both photolabeled residues face intersubunit clefts,[9,10] and subunit stoichiometry studies indicate that each receptor contains two α/β interfacial pockets.[11,12] $GABA_A$ receptor mutations to bulky hydrophobic tryptopan (W) at either α1M236 or β2M286 mimic etomidate's effects by reducing GABA EC_{50}, increasing spontaneous activity, and increasing maximal GABA efficacy, while reducing modulation by etomidate.[13] Together, these results support the hypothesis that etomidate binds within transmembrane interfacial pockets between α-M1 and β-M3 domains, and that there are two etomidate sites on each $\alpha1\beta2\gamma2$ receptor (fig. 1).

Current models of etomidate action assume that the two binding sites have identical structure and contribute equivalently and independently to modulation of receptor gating,[7] but these features remain untested. Asymmetry could be caused by the different spatial relationship of the γ2 subunit to each α/β interfacial etomidate site, which could induce unequal etomidate effects at each site. Furthermore, either positive or negative cooperative interactions may occur between etomidate sites.

In this study, we used constrained receptor assembly to test whether the individual etomidate binding sites produce equivalent and independent effects on receptor function. Concatenated-subunit wild-type dimer ($\beta2$-$\alpha1$ = D^{wt}) and trimer ($\gamma2$-$\beta2$-$\alpha1$ = T^{wt}) polypeptides or concatemers containing α1M236W mutations ($\beta2$-α1M236W = $D^{\alpha M236W}$; $\gamma2$-$\beta2$-α1M236W = $T^{\alpha M236W}$) were coexpressed in *Xenopus* oocytes to produce functional $GABA_A$ receptors with five subunits arranged $\beta2$-$\alpha1$/$\gamma2$-$\beta2$-$\alpha1$ counterclockwise when viewed from the synaptic cleft (fig. 1).[12,14] We compared multiple functional characteristics of all four receptors formed with concatenated dimers and trimers ($D^{wt}T^{wt}$, $D^{\alpha M236W}T^{\alpha M236W}$, $D^{\alpha M236W}T^{wt}$, and $D^{wt}T^{\alpha M236W}$), with particular focus on whether the two single-site mutants, $D^{\alpha M236W}T^{wt}$ and $D^{wt}T^{\alpha M236W}$, displayed equivalent *versus* nonequivalent properties. To determine if etomidate sites interact cooperatively, we also tested whether adding together $D^{\alpha M236W}T^{wt}$ and $D^{wt}T^{\alpha M236W}$ effects fully accounted for the large differences in GABA and etomidate sensitivities between $D^{wt}T^{wt}$ receptors and $D^{\alpha M236W}T^{\alpha M236W}$ receptors.

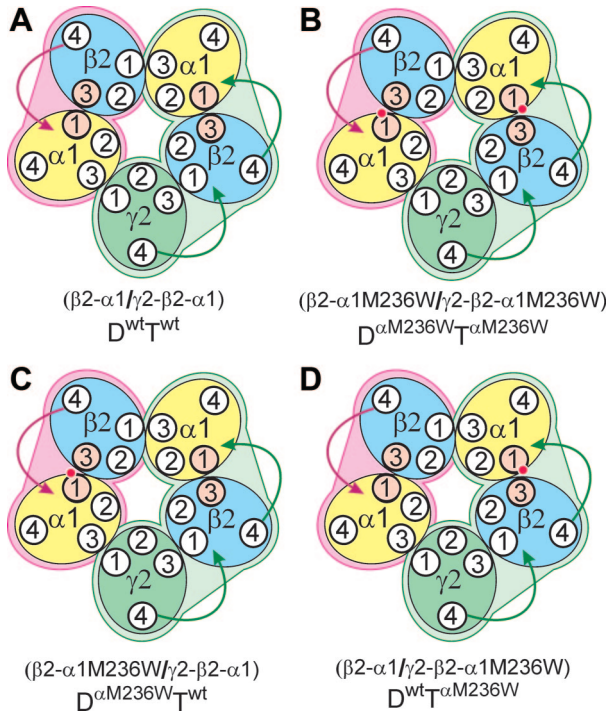

Fig. 1. γ-Aminobutyric acid receptors type A ($GABA_A$) formed from $\beta2$-$\alpha1$ dimers and $\beta2$-$\alpha1$-$\gamma2$ trimers. Each diagram illustrates the structure of one of the four $GABA_A$ receptors used for our experiments, labeled two ways. Labels in parentheses list the concatemer peptide components from amino-terminus to carboxy-terminus, including subunits and linkers (*dashes*), with *forward slashes* between dimers and trimers. Additional labels use the abbreviated nomenclature we have adopted for this study. Channels are (*A*) $D^{wt}T^{wt}$, (*B*) $D^{\alpha M236W}T^{\alpha M236W}$, (*C*) $D^{\alpha M236W}T^{wt}$, and (*D*) $D^{wt}T^{\alpha M236W}$. The arrangement of α1 (*yellow*), $\beta2$ (*blue*), and $\gamma2$ (*green*) $GABA_A$ receptors subunits, as viewed from the extrasynaptic space, is depicted, along with the positions of M1, M2, M3, and M4 transmembrane domains (*numbered circles*) within each subunit. Transmembrane domains forming etomidate sites (α1-M1 and $\beta2$-M3) are shaded *light pink*. Polypeptide linkers between subunits are shown as arrows starting at M4 (near the subunit carboxy-terminus) and connecting to the mature amino-terminus of the following subunit. The $\beta2$-$\alpha1$ dimers are identified by *pink* shaded backgrounds, and the $\beta2$-$\alpha1$-$\gamma2$ trimers by *green* shaded backgrounds. *Red dots* represent the approximate position of αM236W mutations in each type of channel. Note the different spatial relationship between the $\gamma2$ subunit and the distinct etomidate sites formed within dimer and trimer polypeptides. A different subunit is positioned between $\gamma2$ and each etomidate site, and these interposed subunits also abut different sides of $\gamma2$.

Methods and Materials

Animal Use

Oocytes for electrophysiology were harvested from *Xenopus laevis* frogs, as described previously.[7,13] Animals were housed in a veterinarian-supervised facility and used with approval of the Massachusetts General Hospital Subcommittee on Research and Animal Care, in accordance with federal and institutional guidelines.

Materials

Plasmids encoding the concatenated rat dimer ($\beta2$-$\alpha1$) and trimer ($\gamma2$-$\beta2$-$\alpha1$) subunit proteins were a gift from Erwin Sigel, Ph.D. (Professor, Institute for Biochemistry & Molecular Medicine, University of Bern, Bern, Switzerland).[12,14] These plasmids encode polypeptides that insert a 26-residue linker (QQQQQAAAPAQQAAAPAAQQQQQ) between the C-terminus of $\beta2$ and the N-terminus of the mature $\alpha1$ (without its leader sequence). The trimer also contains a 23-residue linker (QQQQQAAAPAQQQAQAAAPAAQ

QQQQ) between the C-terminus of γ2 and the N-terminus of the mature β2. DNA mutations encoding a tryptophan substitution at residue 236 of the mature α1 sequence (α1M236W) were introduced into plasmids using Quickchange (Stratagene, La Jolla, CA), as described previously,[13] and confirmed by complete DNA sequencing of the coding regions. The αM236W mutation was chosen because it impacts multiple receptor function parameters (spontaneous activity, GABA EC_{50}, GABA efficacy, etomidate modulation, and etomidate direct activation), and we aimed to determine how single etomidate site mutations affect each of these parameters. R-etomidate was obtained from Bedford Laboratories (Bedford, OH) as a 2 mg/ml clinical formulation in 35% propylene glycol:water (v/v). GABA and other chemicals (more than 99% pure) were purchased from Sigma-Aldrich (St. Louis, MO).

$GABA_A$ Receptor Expression in Xenopus Oocytes

Messenger RNA was synthesized on linearized coding DNA templates using commercial kits for both DNA transcription and polyadenylation (Ambion, Austin, TX). After purification, mixtures of messenger RNAs (dimer:trimer molar ratio = 1:1) were injected into *Xenopus* oocytes, which were then incubated at 18°C for 1–3 days. All four receptor types formed by combining wild-type or mutant dimers and trimers, $D^{wt}T^{wt}$, $D^{\alpha M236W}T^{wt}$, $D^{wt}T^{\alpha M236W}$, and $D^{\alpha M236W}T^{\alpha M236W}$ (fig. 1), were studied electrophysiologically.

Oocyte Electrophysiology

Receptor-mediated currents were measured in oocytes at 21–22°C using the two-electrode voltage clamp (Model OC-725, Warner Instruments) technique. Perfusion and data acquisition were computer-controlled using Clampex8.1 software *via* a Digidata 1440 interface (both from Molecular Devices, Cupertino, CA). During electrophysiological experiments, oocytes were placed in a 30 μl flow-chamber and perfused at a rate of 3 ml/min with recording buffer ND96 (96 mM NaCl, 2 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.5). GABA or etomidate solutions in ND96 were applied until currents reached a steady maximum or displayed desensitization. Wash-out times ranged from 1 min (low GABA concentrations) to 5 min (high GABA or low etomidate concentrations), or 10 min (high etomidate concentrations). In different sets of oocytes, GABA concentration-responses were measured either in the absence or presence of etomidate (3.2 μM, for comparison with previous studies of receptors formed from free subunits).[7,13] Picrotoxin (2 mM in ND96), a potent $GABA_A$ receptor inhibitor, was used to assess spontaneous channel activity. Maximal GABA efficacy was estimated in two ways. For etomidate-sensitive channels ($D^{wt}T^{wt}$, $D^{\alpha M236W}T^{wt}$, and $D^{wt}T^{\alpha M236W}$), the response to maximal GABA (1–3 mM) was compared with responses with maximal GABA plus etomidate (3.2 μM). For $D^{\alpha M236W}T^{\alpha M236W}$ channels that displayed very weak etomidate modulation, alphaxalone (10 μM) was shown to enhance (at least fivefold) currents elicited with low (EC_{10-20}) GABA. $D^{\alpha M236W}T^{\alpha M236W}$ receptor currents activated with maximal GABA (0.1 mM) were compared in the same cells with currents elicited with maximal GABA plus alphaxalone. We presume that maximal GABA plus the positive modulators activate all receptors.

Statistical Analysis

Peak currents were digitally filtered (lowpass Gaussian 5–10 Hz), baseline corrected, and measured offline using Clampfit software (Molecular Devices). All currents were normalized to maximal GABA response in the same cell, which was measured at the beginning and intermittently throughout experiments on each oocyte. At least four measurements in different cells were made for each type of experiment and experimental condition. Normalized current data from all cells in each type of experiment were combined for nonlinear least-squares method analysis. Agonist concentration-response curves were fitted to logistic (Hill) functions with variable slope (eq. 1) using Graphpad Prism 5.1 (La Jolla, CA):

$$I^{Agonist} = \frac{I_{max} - I_{min}}{1 + 10^{(\log EC_{50} - \log[Agonist])*nH}} + I_{min} \quad (1)$$

Agonist is either GABA or etomidate, EC_{50} is the concentration eliciting a current halfway between minimum and maximum, and nH is the Hill slope.

For the three mutated channels, apparent Gibbs free energy shifts (ΔG) for GABA-induced gating were calculated from fitted log (GABA EC_{50}) values associated with mutant *versus* wild-type ($D^{wt}T^{wt}$) concatenated channels (eq. 2):

$$\Delta G = RT \ln\left[\frac{GABA \cdot EC_{50}^{Mutant}}{GABA \cdot EC_{50}^{wt}}\right] = RT \ln(10) \times (\log[GABA \cdot EC_{50}^{Mutant}] - \log[GABA \cdot EC_{50}^{wt}]) \quad (2)$$

R is the universal gas constant (8.314 J/mol · K) and T is absolute temperature.

For all four channel types, etomidate modulation of GABA currents was assessed as the ratio of GABA EC_{50} in the presence of 3.2 μM etomidate to control GABA EC_{50}. Etomidate modulation was also quantified as changes in Gibbs free energy, using the fitted GABA EC_{50} values in the absence and presence of etomidate (eq. 3):

$$\Delta G = RT \ln\left[\frac{GABA \cdot EC_{50}^{ETO}}{GABA \cdot EC_{50}}\right] = RT \ln(10) \times (\log[GABA \cdot EC_{50}^{ETO}] - \log[GABA \cdot EC_{50}]) \quad (3)$$

Maximal direct activation by etomidate was assessed as response to 300 μM etomidate, normalized to maximal GABA response. Higher etomidate concentrations produce a second, inhibitory effect on $GABA_A$ receptors.

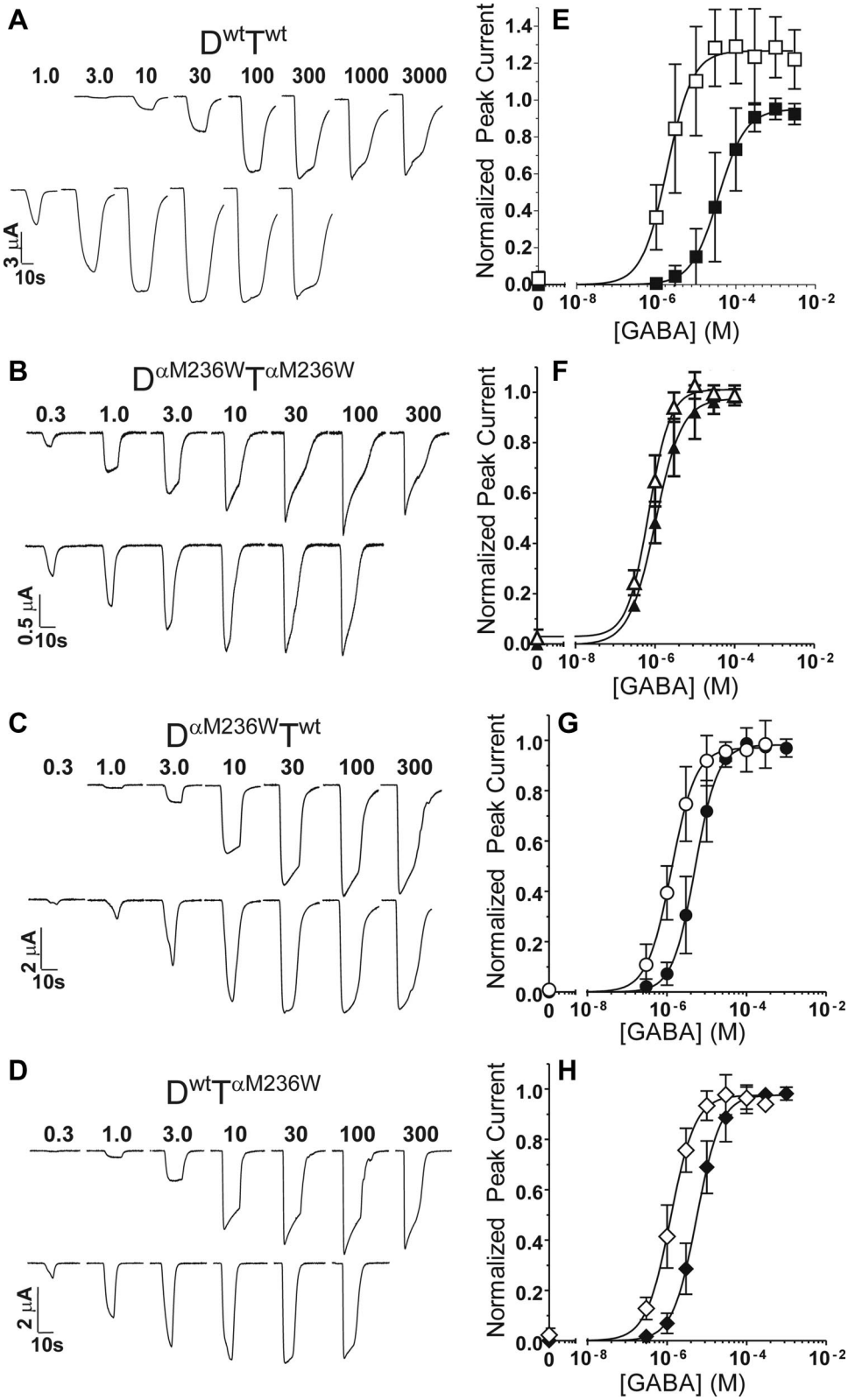

Fig. 2. γ-Aminobutyric acid (GABA) concentration-responses in the absence and presence of etomidate. Results for different pairs of concatenated $GABA_A$ receptor subunit assemblies are shown as pairs of panels: $D^{wt}T^{wt}$ (*A* and *E*), $D^{\alpha M236W}T^{\alpha M236W}$ (*B* and *F*), $D^{\alpha M236W}T^{wt}$ (*C* and *G*), and $D^{wt}T^{\alpha M236W}$ (*D* and *H*). *A–D* display examples of current traces recorded from oocytes using two-electrode voltage clamp. Within each panel, control GABA responses are shown in the top row of traces, and responses in the presence of 3.2 μM etomidate are shown in the bottom row of traces. Each row of traces was recorded from a single oocyte.

Table 1. Fitted Parameters for γ-Aminobutric acid and Etomidate Effects

	$D^{wt}T^{wt}$	$D^{\alpha M236W}T^{\alpha M236W}$	$D^{\alpha M236W}T^{wt}$**	$D^{wt}T^{\alpha M236W}$**
Log(GABA EC_{50})	−4.44 ± 0.078 (−4.59–4.28)	−5.99 ± 0.050 (−6.09–5.89)*	−5.29 ± 0.032 (−5.36–5.23)*	−5.26 ± 0.032 (−5.32–5.19)*
GABA EC_{50} (μM)	36 (25–52)	1.02 (0.81–1.30)*	5.1 (4.39–5.90)*	5.5 (4.79–6.43)*
Hill slope (no etomidate)	1.3 ± 0.24 (0.79–1.76)	1.3 ± 0.18 (0.94–1.67)	1.5 ± 0.14 (1.25–1.81)	1.4 ± 0.12 (1.19–1.69)
Log(GABA EC_{50}^{ETO})	−5.72 ± 0.088 (−5.90–5.54)	−6.16 ± 0.036 (−6.24–6.09)*	−5.89 ± 0.047 (−5.98–5.79)	−5.91 ± 0.037 (−5.99–5.84)
GABA EC_{50}^{ETO} (μM)	1.9 (1.25–2.87)	0.69 (0.58–0.82)*	1.3 (1.04–0.62)	1.2 (1.03–1.46)
Max (*vs.* I_{GABA}^{max})	1.27 ± 0.053 (1.16–1.38)	1.01 ± 0.017 (0.98–1.05)*	0.97 ± 0.024 (0.92–1.02)*	0.98 ± 0.021 (0.93–1.02)*
Hill slope (with etomidate)	1.4 ± 0.39 (0.56–2.17)	1.6 ± 0.17 (1.25–1.96)	1.4 ± 0.20 (1.01 to 1.84)	1.4 ± 0.17 (1.06–1.75)
Left-shift ratio	19 (11.2–33.1)	1.5 (1.12–1.97)*	3.9 (3.02–5.10)*	4.5 (3.61–5.64)*
Apparent GABA Efficacy	0.79 ± 0.032 (0.73–0.86)	0.99 ± 0.016 (0.95–1.03)*	1.03 ± 0.025 (0.98–1.07)*	1.02 ± 0.021 (0.98–1.06)*
Log(ETO EC_{50})	−4.22 ± 0.050 (−4.36–4.08)	−4.69 ± 0.076 (−4.85–4.54)*	−4.28 ± 0.020 (−4.34–4.23)	−4.39 ± 0.045 (−4.52–4.26)
ETO EC_{50} (μM)	61 (44.1–83.1)	20 (14.1–29.1)*	52 (46.2–59.4)	41 (30.6–54.6)
ETO Hill slope	1.4 ± 0.08 (1.18–1.64)	1.3 ± 0.30 (0.64–1.88)	1.4 ± 0.10 (1.20–1.61)	1.8 ± 0.12 (1.55–2.20)
ETO Efficacy (*vs.* I_{GABA}^{max})	0.16 ± 0.0077 (0.14–0.19)	0.53 ± 0.032 (0.47–0.60)*	0.27 ± 0.0063 (0.26–0.29)*	0.31 ± 0.014 (0.27–0.35)*
Spontaneous Activity	<0.001	<0.001	<0.001	<0.001

Fitted parameters are reported as mean ± SD (95% CI). Parameters calculated from fitted log(EC_{50}) values are reported as mean (95% CI). * Parameter differs from $D^{wt}T^{wt}$ parameter at $P < 0.015$. ** Parameters for $D^{\alpha M236W}T^{wt}$ and $D^{wt}T^{\alpha M236W}$ channels are indistinguishable ($P > 0.15$ for all comparisons).

ETO = etomidate; GABA = γ-aminobutyric acid.

Results are reported as mean ± SD unless otherwise noted. Standard errors for fitted log(EC_{50}) values were used to calculate 95% CIs for EC_{50}s. Comparisons of concentration-responses in the presence and absence of etomidate and for different receptors were performed using the comparison function in the nonlinear fitting module of Graphpad Prism. Statistical comparison of etomidate-induced shifts for different types of channels were performed by calculating the z-statistic from log(EC_{50}) differences. Other statistical analyses were performed in Graphpad Prism or Microsoft Excel (Redmond, WA) using one-way ANOVA with Tukey *post hoc* test. For pairwise comparisons (*e.g.*, GABA concentration-responses in the presence *vs.* absence of etomidate) we used $P < 0.05$ to establish significance. For multiple comparisons of each receptor type to three others, we applied Bonferroni correction, and used $P < 0.015$ to establish significance.

Results

GABA Concentration-responses and EC_{50}s

All four messenger RNA combinations of dimers (D^{wt} or $D^{\alpha M236W}$) and trimers (T^{wt} or $T^{\alpha M236W}$) produced functional GABA-responsive receptors in oocytes, with maximal currents typically over 2 μA at a clamping potential of −50 mV (figs. 2A–D). Oocytes injected with only dimer or only trimer messenger RNA produced maximal currents in response to GABA that were at most 10-fold smaller than those from oocytes coexpressing both dimers and trimers. The wild-type dimer and trimer combination, $D^{wt}T^{wt}$, displayed a GABA EC_{50} of 36 μM (fig. 2E; table 1). Receptors with mutations in both etomidate sites, $D^{\alpha M236W}T^{\alpha M236W}$, showed a much lower GABA EC_{50} of 1.0 μM, (fig. 2F; table 1; $P < 0.0001$ *vs.* $D^{wt}T^{wt}$). The two single-site mutant receptors, $D^{\alpha M236W}T^{wt}$ and $D^{wt}T^{\alpha M236W}$, were characterized by indistinguishable GABA EC_{50} values: 5.1 and 5.5 μM, respectively (figs. 2G and 2H; table 1; $P = 0.53$). These values differed significantly from GABA EC_{50}s for both $D^{wt}T^{wt}$ and $D^{\alpha M236W}T^{\alpha M236W}$ ($P < 0.0001$ for all pairwise comparisons). Plotting all four GABA concentration responses on a single semilogarithmic plot reveals that the midpoints (EC_{50}) for both single-site mutant channels are approximately halfway between those for $D^{wt}T^{wt}$ and $D^{\alpha M236W}T^{\alpha M236W}$ channels (fig. 3A). Calculating the Gibbs free energy for GABA EC_{50} shifts associated with the different mutated channels relative to $D^{wt}T^{wt}$ (eq. 2) reveals that the single-site mutants, $D^{\alpha M236W}T^{wt}$ and $D^{wt}T^{\alpha M236W}$, contribute −4.9 ± 0.48 and −4.7 ± 0.48 kJ/mol, respectively, each about half of the energy shift (−8.9 ± 0.52 kJ/mol) calculated for $D^{\alpha M236W}T^{\alpha M236W}$ channels (fig. 3B).

Etomidate Modulation of GABA Responses

To assess etomidate modulation of $GABA_A$ receptor activation, we measured GABA-dependent responses in the pres-

Fig. 2. (Continued) Labels above the traces show GABA concentrations (μM) used to elicit currents. *E–H* display GABA concentration-responses in the absence or presence of etomidate, normalized to the maximal GABA response. Each *symbol* represents mean ± SD of at least four independent measurements from different oocytes, but full concentration-response data sets were not obtained in every oocyte. *Solid symbols* represent control GABA responses and open symbols represent GABA responses in the presence of 3.2 μM etomidate. *Lines* through data represent fits to logistic functions (eq. 1, see Materials and Methods). Fitted parameters for each type of receptor are reported in table 1. Marked in panels *E–H* are $D^{wt}T^{wt}$ control (*E*, *solid squares*); $D^{wt}T^{wt}$ + etomidate (*E*, *open squares*); $D^{\alpha M236W}T^{\alpha M236W}$ control (*F*, *solid triangles*); $D^{\alpha M236W}T^{\alpha M236W}$ + etomidate (*F*, *open triangles*); $D^{\alpha M236W}T^{wt}$ control (*G*, *solid circles*); $D^{\alpha M236W}T^{wt}$ + etomidate (*G*, *open circles*); $D^{wt}T^{\alpha M236W}$ control (*H*, *solid diamonds*); $D^{wt}T^{\alpha M236W}$ + etomidate (*H*, *open diamonds*). GABA = γ-aminobutyric acid.

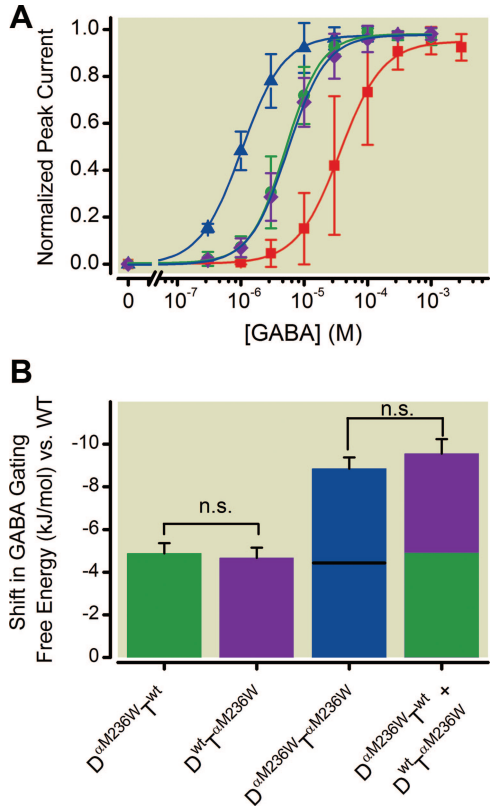

Fig. 3. Mutant-associated changes in γ-aminobutyric acid (GABA) sensitivity display energy additivity. (*A*) GABA concentration response data from figure 2 are redrawn on a single set of axes to highlight the comparative responses for all four channel types: $D^{wt}T^{wt}$ (*red squares*); $D^{\alpha M236W}T^{\alpha M236W}$ (*blue triangles*); $D^{\alpha M236W}T^{wt}$ (*green circles*); $D^{wt}T^{\alpha M236W}$ (*purple diamonds*). *Lines* through data represent fits to logistic functions (eq. 1, see Materials and Methods). Fitted parameters are reported in table 1. (*B*) For the three mutant channels, the change in GABA gating energy relative to $D^{wt}T^{wt}$ channels was calculated (eq. 2, see Materials and Methods) and is depicted in a bar graph for the two single-mutant channels, $D^{\alpha M236W}T^{wt}$ (*green*) and $D^{wt}T^{\alpha M236W}$ (*purple*), and the double mutant, $D^{\alpha M236W}T^{\alpha M236W}$ (*blue*). The *black line* indicates half of the double-mutant shift energy. A fourth bar (*green and purple*) depicts the sum of the shift energies associated with the two single-mutant channels. GABA = γ-aminobutyric acid; n.s. = not significant ($P > 0.015$).

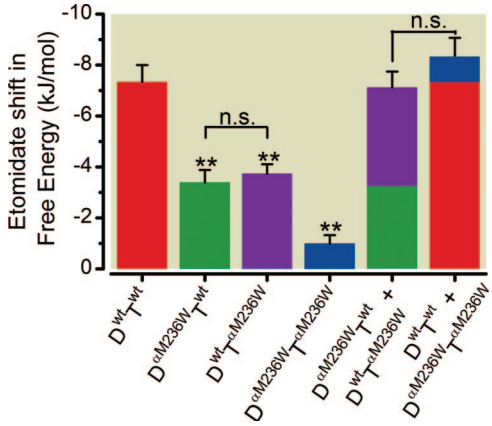

Fig. 4. Energy additivity of mutant-associated changes in etomidate modulation. For all four channel types, γ-aminobutyric acid (GABA) response left-shift ratios at 3.2 μM etomidate were used to calculate apparent shift energy in kJ/mol (eq. 3, see Materials and Methods). These are depicted in a bar graph: $D^{wt}T^{wt}$ (*red*), $D^{\alpha M236W}T^{wt}$ (*green*), $D^{wt}T^{\alpha M236W}$ (*purple*) and the double mutant, $D^{\alpha M236W}T^{\alpha M236W}$ (*blue*). Energy additivity was assessed by comparing the sum of the two single-site mutant shifts (*green* and *purple stacked bars*) with the sum of the wild-type and double-mutant shifts (*red* and *blue stacked bars*). ** indicates significantly different from wild-type ($P < 0.005$). n.s. = no significant difference ($P > 0.015$).

ence of 3.2 μM etomidate (figs. 2E–H, open symbols) and for each channel type calculated the ratios of GABA EC_{50}s measured in the absence and presence of anesthetic (Left Shift Ratios, table 1). In $D^{wt}T^{wt}$ receptors, etomidate lowered the GABA EC_{50} 19-fold, from 36 to 1.9 μM ($P < 0.0001$), whereas $D^{\alpha M236W}T^{\alpha M236W}$ GABA EC_{50} was reduced only 1.5-fold, from 1.0 to 0.69 μM ($P = 0.0065$). The EC_{50} ratios in $D^{wt}T^{wt}$ *versus* $D^{\alpha M236W}T^{\alpha M236W}$ were significantly different ($P < 0.0001$). Etomidate shifted GABA EC_{50}s 3.9-fold, from 5.1 to 1.3 μM, in $D^{\alpha M236W}T^{wt}$ receptors ($P < 0.0001$), and 4.5-fold, from 5.5 to 1.2 μM, in $D^{wt}T^{\alpha M236W}$ receptors ($P < 0.0001$). The two single-site mutant left-shift ratios were not significantly different from each other ($P = 0.21$), but they differed significantly from values for both $D^{wt}T^{wt}$ ($P < 0.0001$) and $D^{\alpha M236W}T^{\alpha M236W}$ ($P < 0.0001$). Figure 4 shows etomidate left-shifts expressed as free energy changes, calculated with eq. 3. This display reveals that adding the gating free energy change associated with etomidate modulation in $D^{\alpha M236W}T^{wt}$ and $D^{wt}T^{\alpha M236W}$ receptors (−3.4 ± 0.49 and −3.7 ± 0.38 kJ/mol, respectively) sum to a value (−7.1 ± 0.62 kJ/mol) that is not significantly different ($P = 0.11$) from the sum of free energy changes for $D^{wt}T^{wt}$ plus $D^{\alpha M236W}T^{\alpha M236W}$ (−8.3 ± 0.75 kJ/mol).

Etomidate Direct Activation

In the absence of GABA, etomidate directly activated all four receptors (fig. 5; table 1). Maximal etomidate currents in $D^{wt}T^{wt}$ channels, elicited with 100–300 μM anesthetic, were 16% of currents stimulated with maximal GABA, and the fitted half-maximal activation (EC_{50}) etomidate concentration was 61 μM. $D^{\alpha M236W}T^{\alpha M236W}$ receptors displayed greater sensitivity to etomidate direct activation, with half-maximal activation at 20 μM ($P < 0.0001$ *vs.* $D^{wt}T^{wt}$) and maximal efficacy relative to GABA of 53% ($P < 0.0001$ *vs.* $D^{wt}T^{wt}$). Etomidate activation studies in $D^{\alpha M236W}T^{wt}$ and $D^{wt}T^{\alpha M236W}$ receptors revealed similar etomidate EC_{50}s (52 *vs.* 41 μM; $P = 0.38$) and similar maximal etomidate efficacies (27 *vs.* 31%; $P = 0.16$). The maximal etomidate activation efficacies of the $D^{\alpha M236W}T^{wt}$ and $D^{wt}T^{\alpha M236W}$ channels were both significantly higher than that of $D^{wt}T^{wt}$ and significantly lower than that of $D^{\alpha M236W}T^{\alpha M236W}$ ($P <$

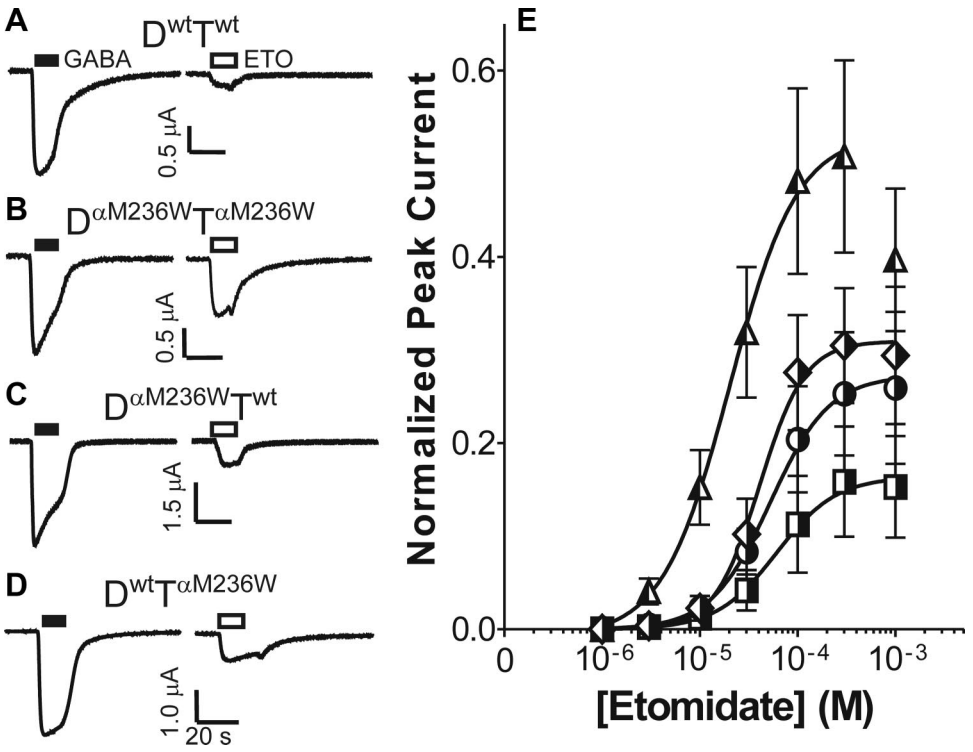

Fig. 5. Etomidate direct activation concentration responses. (*A–D*) Four pairs of current traces, recorded from *Xenopus* oocytes expressing different concatenated channels (labeled), are shown. Paired currents are each from a single oocyte, and were elicited with either maximal γ-aminobutyric acid (GABA) (1–3 mM) or maximal etomidate (0.3 mM). (*E*) Concentration-response data (mean ± SD; n ≥ 4) for etomidate direct activation are shown for all four types of channels, normalized to maximal GABA responses: $D^{wt}T^{wt}$ (*half-solid squares*), $D^{\alpha M236W}T^{\alpha M236W}$ (*half-solid triangles*), $D^{\alpha M236W}T^{wt}$ (*half-solid circles*), and $D^{wt}T^{\alpha M236W}$ (*half-solid diamonds*). *Lines* through data points are nonlinear least-squares fit to logistic functions (eq. 1, see Materials and Methods). The 1 mM etomidate data were excluded from the fit for $D^{\alpha M236W}T^{\alpha M236W}$ receptors, because a second inhibitory effect of etomidate was evident. Fitted logistic parameters are reported in table 1.

than 0.01 for all pairs by one-way ANOVA). Etomidate EC_{50}s for $D^{\alpha M236W}T^{wt}$ and $D^{wt}T^{\alpha M236W}$ channels were similar to that for $D^{wt}T^{wt}$ ($P = 0.84$ and 0.21, respectively), but significantly higher than that for $D^{\alpha M236W}T^{\alpha M236W}$ ($P < 0.001$).

Receptor Spontaneous Activity and Maximal GABA Efficacy

Spontaneous activity in $GABA_A$ receptors was assessed using the noncompetitive antagonist picrotoxin. When present, spontaneously open channels produce a resting-state current "leak," which is inhibited by picrotoxin, resulting in an apparently outward current.[7,13,15] None of the concatenated $GABA_A$ dimer and trimer combinations displayed measurable picrotoxin-induced outward currents. Given a maximal GABA signal-to-noise ratio of about 1,000 in these experiments, these results indicate that spontaneous activity is less than 0.1% of maximal GABA response (table 1).

The maximal GABA efficacy (table 1) represents the fraction of activatable receptors that open when all agonist sites are bound to GABA. GABA efficacy in $D^{wt}T^{wt}$ receptors was 0.79, based on etomidate enhancement of 3 mM GABA responses (fig. 2E). In contrast, etomidate did not significantly enhance maximal (1 mM) GABA responses in either $D^{\alpha M236W}T^{wt}$ or $D^{wt}T^{\alpha M236W}$ receptors (figs. 2G and 2H), indicating maximal GABA efficacies near 1.0. In $D^{\alpha M236W}T^{\alpha M236W}$ receptors, which displayed low sensitivity to etomidate, alphaxalone was a strong allosteric enhancer at low GABA, but did not significantly enhance responses to maximal (0.1 mM) GABA (not shown). Thus, we infer that the maximal GABA efficacy in $D^{\alpha M236W}T^{\alpha M236W}$ channels is near 1.0. All mutant channels displayed GABA efficacies significantly higher than $D^{wt}T^{wt}$ ($P < 0.001$ for all pairs by one-way ANOVA).

Discussion

We coexpressed concatenated $GABA_A$ subunit β2-α1 dimers and γ2-β2-α1 trimers that formed functional α1β2γ2 l receptors containing α1M236W mutations in neither, either, or both of the two etomidate sites formed between α1-M1 and β2-M3 domains. The major finding of this study is that a single α1M236W mutation in either the dimer or trimer etomidate binding site produces receptors with equivalent functional characteristics. With respect to both GABA sensitivity and etomidate modulation, each site contributes about half the effect observed in wild-type receptors with two etomidate sites.

Implicit in our experimental design is the assumption that subunit concatenation itself does not significantly alter the interaction of etomidate with $GABA_A$ receptors. Concatenated wild-type control receptors assembled from $D^{wt}T^{wt}$ displayed etomidate sensitivity similar to that previously reported in wild-type $\alpha1\beta2\gamma2$ channels formed with free $\alpha1$, $\beta2$, and $\gamma2$ subunits. Etomidate at 3.2 μM reduced GABA EC_{50} 19-fold in $D^{wt}T^{wt}$ receptors, comparable with shifts ranging from 11-fold to 23-fold previously reported for $\alpha1\beta2\gamma2$ l receptors.[7,13,16] Etomidate direct agonism in $D^{wt}T^{wt}$ was characterized by EC_{50} and efficacy parameters similar to those reported for $\alpha1\beta2\gamma2$ channels by Rüsch *et al.*[7]

The effects of two $\alpha1$M236W mutations in the concatenated receptors ($D^{\alpha M236W}T^{\alpha M236W}$) also paralleled effects previously reported in $\alpha1$M236W$\beta2\gamma2$ l $GABA_A$ receptors formed from free subunits.[13] In $D^{\alpha M236W}T^{\alpha M236W}$ receptors, GABA EC_{50} was reduced 35-fold relative to wild-type ($D^{wt}T^{wt}$), and the GABA EC_{50} ratio was 1.5-fold at 3.2 μM etomidate. In receptors formed from free subunits, $\alpha1$M236W mutations reduced GABA EC_{50} 20-fold, and GABA EC_{50} ratio with 3.2 μM etomidate was 1.7-fold.[13] Another similarity to studies in $GABA_A$ receptors with free subunits is that etomidate direct activation in $D^{\alpha M236W}T^{\alpha M236W}$ channels was more potent and efficacious than in $D^{wt}T^{wt}$. These effects are consistent with previous analysis[7,13] suggesting that $\alpha1$M236W mutations produce a loss-of-function with respect to etomidate efficacy, combined with increased basal gating. The former explains the reduced etomidate left shift, whereas the latter underlies the increased sensitivity to orthosteric agonism by GABA and allosteric agonism by etomidate.[13] In brief, tryptophan mimics the presence of etomidate, increasing GABA sensitivity and efficacy, while also reducing etomidate's effectiveness as a modulator. Although photolabeling indicates that αM236 is near bound etomidate, we do not know precisely how the tryptophan mutation alters etomidate binding interactions. Based on the relative size of methionine and tryptophan sidechains, a steric component is postulated.

Calculating the gating free energy changes associated with one *versus* two $\alpha1$M236W mutations reveals that each mutation contributes equally and additively. Both single-mutant receptors ($D^{\alpha M236W}T^{wt}$ and $D^{wt}T^{\alpha M236W}$) displayed indistinguishable GABA EC_{50}s and each mutation accounted for approximately half of the GABA gating energy change calculated for $D^{\alpha M236W}T^{\alpha M236W}$ (fig. 3; table 1). Similarly, $D^{\alpha M236W}T^{wt}$ and $D^{wt}T^{\alpha M236W}$ displayed indistinguishable etomidate modulation, with each site contributing approximately half of the total energy change associated with etomidate modulation in $D^{wt}T^{wt}$ receptors (fig. 4; table 1). These experiments also demonstrate that a single etomidate site is sufficient for receptor modulation to occur.

In addition to the results discussed above, we found that etomidate direct activation, spontaneous activation, and maximal GABA efficacy were all indistinguishable in receptors with one $\alpha1$M236W mutation in either the dimer or trimer (fig. 5; table 1). Thus, the two sites have indistinguishable structures and interactions with etomidate molecules, and the $\gamma2$ subunit does not produce significant asymmetrical effects on the etomidate sites.

We can infer from our free energy calculations whether cooperativity between the two etomidate sites is present. If positive cooperativity between two equivalent sites exists, each single-site mutant (with one intact site) would display significantly less than half of the etomidate-induced energy shift observed in wild-type receptors, whereas negative cooperativity would produce converse results. The energy additivity found for both GABA gating and etomidate shift effects indicates that cooperativity between etomidate sites is negligible.

Previous studies using $GABA_A$ subunit concatemers and loss-of-function mutations for various ligands provide additional evidence for symmetry in transmembrane modulator sites, whereas studies of the extracellular agonist sites reveal some asymmetrical effects.[17,18] Neuroactive steroids potentiate GABA currents and directly activate receptors at high concentrations, effects that are reduced by mutations at α_1Q241.[19] In concatenated $\gamma2$-$\beta2$-$\alpha1$ trimers and $\beta2$-$\alpha1$ dimers, single $\alpha1$Q241 mutations produced similar functional effects when introduced into either peptide.[17] Orthosteric agonists and competitive antagonists bind at two extracellular α/β interfacial sites.[1,18] Single βY205S GABA-site mutations, which produce profound loss of agonist function, produced asymmetrical effects on GABA and muscimol agonist EC_{50}s, and symmetrical effects on bicuculline IC_{50}.[18] These studies also showed that for efficient channel activation, both GABA sites must be occupied, whereas antagonist binding to either GABA site effectively blocks activation.

Some of our studies characterizing $D^{\alpha M236W}T^{\alpha M236W}$ receptor function revealed differences from $\alpha1$M236W$\beta2\gamma2$ $GABA_A$ receptors formed from free subunits, which displayed both spontaneous activity and full agonism by etomidate.[13] Several factors may contribute to these differences. First, our concatenated $GABA_A$ receptors contain rat subunits, whereas previous studies on $\alpha1$M236W mutations were performed using human $GABA_A$ subunits with slightly different amino acid sequences. Second, receptors formed from concatemers are constrained to include a γ subunit, whereas evidence suggests that γ subunits are not consistently incorporated into receptors when free subunits are coexpressed in *Xenopus* oocytes.[20,21] Finally, GABA EC_{50}s in concatenated $GABA_A$ receptors have been reported to be higher than receptors formed from identical free subunits, suggesting that concatenation itself may reduce basal gating.[12,22]

In studies using concatenated channel subunit assemblies it is important to verify that linked subunits are not enzymatically cleaved into free subunits, and in the present case, to demonstrate that expression of dimers alone or trimers alone does not lead to significant formation of alternative channel assemblies that might influence results.[22] Our control exper-

iments showed that expression of dimers alone or trimers alone produced small GABA-responsive currents, with amplitudes less than 10% of those recorded when dimers and trimers were coexpressed. Thus, alternative concatemer channel assemblies are at most a minor contributor to our electrophysiological measurements. Concatemer cleavage leading to assembly of receptors from free subunits also appears to be insignificant, based on these observations. The high Hill slopes observed in GABA concentration responses for $D^{\alpha M236W}T^{wt}$ and $D^{wt}T^{\alpha M236W}$ receptors (figs. 2, 3) is further evidence against concatemer breakdown. If free subunits were produced, mixtures of receptors containing zero, one, or two α1M236W mutations would form, resulting in shallow (low Hill slope) GABA concentration-response curves reflecting the dose-dependent gating effects of the mutation.[11]

The experiments reported here did not address whether a single etomidate site is capable of directly activating wild-type $GABA_A$ receptors. We observed etomidate direct activation in $D^{\alpha M236W}T^{wt}$ and $D^{wt}T^{\alpha M236W}$ receptors, but this is likely because of the combined effects of one intact etomidate site together with basal gating enhancement associated with one α1M236W mutation, which contributes gating energy similar to partial etomidate occupancy. A related study using concatemers combining etomidate-sensitive β2 and etomidate-insensitive β1 subunits in the same $GABA_A$ receptors suggests that a single β2 subunit is not sufficient for direct activation with etomidate.[23] This question could be further addressed with concatemer studies using point mutations that selectively and profoundly reduce etomidate sensitivity.

In conclusion, our results show that etomidate interactions at each of its two $GABA_A$ receptor sites produce equal and additive gating modulation effects. These results support functional models wherein the two etomidate sites in $\alpha1\beta2\gamma2$ l receptors are equivalent and noncooperative.[7] In terms of clinical relevance, these studies add important details to our understanding of anesthetic mechanisms at their molecular targets, a key step toward developing improved anesthetic drugs.[24] Of more direct relevance, heteromeric $GABA_A$ receptors containing both β1 and β2/3 subunits are postulated to exist in the nervous system.[23] In the presence of etomidate, our results predict that such receptors will experience approximately half the gating energy effect of receptors containing two β2/3 subunits. Future studies using concatenated $GABA_A$ subunit assemblies will be useful in investigating the linkages between the etomidate and GABA sites on the same subunit, and assessing the mechanisms of other anesthetics, which also likely bind to multiple sites.[25]

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Codrenin

Detroit's Parke, Davis & Company released Codrenin (*above*) as "a local anesthetic and hemostatic" consisting of a 2% solution of cocaine mixed with adrenaline. Since both chemicals were vasoconstrictors, the mixture worked remarkably well when "painted" on bleeding surfaces. However, when Codrenin was injected, particularly in too large a quantity or intravascularly, clinicians could witness in some patients a spectacular display of cardiovascular instability, seizure activity, and even death. Fortunately, local anesthetics less toxic than cocaine, such as procaine (Novocaine), would eventually be synthesized and would supplant the hazardous combination of adrenaline and cocaine in proprietary mixtures such as Codrenin. (Copyright © the American Society of Anesthesiologists, Inc.)

George S. Bause, M.D., M.P.H., Honorary Curator, ASA's Wood Library-Museum of Anesthesiology, Park Ridge, Illinois, and Clinical Associate Professor, Case Western Reserve University, Cleveland, Ohio. UJYC@aol.com.