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γ-Amino Butyric Acid Type A Receptor Mutations at β2N265 Alter Etomidate Efficacy While Preserving Basal and Agonist-dependent Activity

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Background: **Etomidate acts at γ-Aminobutyric acid type A ($GABA_A$) receptors containing β2 or β3, but not β1 subunits. Mutations at β residue 265 (Ser in β1; Asn in β2 or β3) profoundly affect etomidate sensitivity. Whether these mutations alter etomidate binding remains uncertain.**

Methods: **Heterologously expressed α1β2γ2L $GABA_A$ receptors and receptors with β2(N265S) or β2(N265M) mutations were studied electrophysiologically in both *Xenopus* oocytes and HEK293 cells. Experiments quantified the impact of β2N265 mutations or substituting β1 for β2 on basal channel activation, GABA EC_{50}, maximal GABA efficacy, etomidate-induced leftward shift in GABA responses, etomidate direct activation, and rapid macrocurrent kinetics. Results were analyzed in the context of an established allosteric coagonist mechanism.**

Results: **Mutations produced only small changes in basal channel activity, GABA EC_{50}, maximal GABA efficacy, and macrocurrent kinetics. Relative to wild-type, β2(N265S) reduced etomidate enhancement of apparent GABA affinity six-fold, and it reduced etomidate direct activation efficacy 14-fold. β2(N265M) totally eliminated both etomidate modulation of GABA responses and direct channel activation. Mechanism-based analysis showed that the function of both mutants remains consistent with the allosteric coagonist model and that β2(N265S) reduced etomidate allosteric efficacy five-fold, whereas etomidate-binding affinity dropped threefold. Experiments swapping β2 subunits for β1 indicated that etomidate efficacy is reduced 34-fold, whereas binding affinity drops less than two-fold.**

Conclusions: **Mutations at β2N265 profoundly alter etomidate sensitivity with only small changes in basal and GABA-dependent channel activity. Mutations at the β2N265 residue or replacement of β2 with β1 influence etomidate efficacy much more than binding to inactive receptors.**

ETOMIDATE is a potent intravenous general anesthetic that produces its behavioral effects *via* ionotropic γ-Aminobutyric acid type A ($GABA_A$) receptors, the major inhibitory postsynaptic ion channels in mammalian brain.[1,2] Etomidate slows decay of GABAergic inhibitory postsynaptic currents in neurons and similarly slows deactivation of $GABA_A$ receptor-mediated macrocurrents elicited with brief agonist pulses.[3,4] Etomidate potentiates currents elicited by submaximal GABA, shifting GABA EC_{50} to lower concentrations. High concentrations of etomidate also directly activate $GABA_A$ receptors. In α1β2γ2L $GABA_A$ receptors, these etomidate actions are quantitatively described by an allosteric model with two equivalent coagonist sites linked to channel gating.[5]

$GABA_A$ receptors contain a central chloride ion channel surrounded by five homologous subunits, each with a large amino-terminal extracellular domain, four transmembrane domains (M1-M4), and a large intracellular domain between M3 and M4.[6] The most abundant $GABA_A$ receptor subtype, α1β2γ2L, incorporates 2α, 2β, and 1γ arranged counterclockwise as γ-β-α-β-α when viewed from the synaptic cleft.[7–9] Photolabeling with an etomidate analog, [^{3}H]-azi-etomidate,[10,11] identified two $GABA_A$ receptor residues on adjacent subunits, M286 in the β subunit M3 domain and M236 in the α subunit M1 domain.

The amino acid at position 265 (15′) in the M2 domain of β subunits is also a determinant of etomidate sensitivity. Etomidate modulates mammalian $GABA_A$ receptors containing β2 or β3 subunits, which both have Asn (N) at position 265, while minimally affecting receptors containing β1 subunits, which have Ser (S) at position 265.[12] Ser substitutions for Asn265 in β2 and β3 reduce etomidate sensitivity, whereas receptors containing mutant β1(S265N) subunits become etomidate sensitive.[13–15] In addition, the homolog of β2/3(N265) in the anesthetic-insensitive *drosophila rdl* $GABA_A$ receptor is a Met (M), and mutation of β2/3N265 to Met also dramatically reduces etomidate modulation.[16,17] The β3(N265M) and β2(N265S) mutations have been used in transgenic animal studies probing the role of $GABA_A$ receptors in anesthetic actions *in vivo*.[1,2] Structural models of α1β2γ2L $GABA_A$ receptors,[11,18,19] based on a 4-Å resolution structure of the homologous nicotinic acetylcholine receptor from Torpedo,[20] show β2N265 at the periphery of the etomidate binding pocket.

The aims of this study were to quantitatively define the impact of β2N265 mutations on α1β2γ2L $GABA_A$ receptor function both in the absence and presence of etomidate and also to determine whether mutations at β2N265 affect etomidate binding *versus* its allosteric efficacy. We assessed the effects of β2(N265S) and β2(N265M) mutations on spontaneous channel activity, GABA concentration responses, maximum GABA

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efficacy, etomidate modulation of GABA-dependent activation, and direct channel activation by etomidate in the absence of GABA. Macrocurrent activation, desensitization, and deactivation rates were also measured in wild-type and mutant receptors by using submillisecond GABA concentration jumps. Results were analyzed within the mechanistic framework of allosteric coagonism.[5]

Materials and Methods

Animal Use

Female *Xenopus laevis* were housed in a veterinary-supervised environment and used in accordance with local and federal guidelines and with approval from the Massachusetts General Hospital subcommittee on research and animal care (Boston, Massachusetts). Frogs were anesthetized by immersion in ice-cold 0.2% tricaine (Sigma-Aldrich, St. Louis, MO) before mini-laparotomy for oocyte harvest.

Chemicals

R(+)-Etomidate was obtained from Bedford Laboratories (Bedford, OH). The clinical preparation in 35% propylene glycol was diluted directly into buffer. Previous studies have shown that propylene glycol at the dilutions used for these studies has no effect on $GABA_A$ receptor function.[5] Picrotoxin was purchased from Sigma-Aldrich and dissolved in electrophysiology buffer (2 mM) by prolonged gentle shaking. Alphaxalone was purchased from MP Biomedical (Solon, OH) and prepared as a stock solution in dimethylsulfoxide. Salts and buffers were purchased from Sigma-Aldrich.

Molecular Biology

Complementary DNA sequences for human $GABA_A$ receptor $\alpha1$, $\beta2$, $\beta1$, and $\gamma2L$ subunits were cloned into pCDNA3.1 vectors (Invitrogen, Carlsbad, CA). To create expression plasmids for $\beta2$(N265S), $\beta2$(N265M), and $\alpha1$(L264T) mutants, oligonucleotide-directed mutagenesis was performed on the appropriate wild-type clone using QuickChange kits (Stratagene, La Jolla, CA). Clones from each mutagenesis reaction were sequenced through the entire subunit gene to confirm the presence of the mutation and absence of stray mutations.

Expression of $GABA_A$ Receptors

Messenger RNA was synthesized *in vitro* from linearized DNA templates and purified using commercial kits (Ambion Inc., Austin, TX). Subunit messenger RNAs were mixed at 1α:1β and at least two-fold excess γ to promote homogeneous receptor expression.[21,22] *Xenopus* oocytes were microinjected with 25–50 nl (15–25 ng) of messenger RNA mixture and incubated at 18°C in ND96 (in mM: 96 NaCl, 2 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, pH 7.5) supplemented with gentamicin (0.05 mg/ml) for 24–48 h before electrophysiology. HEK293 cells were cultured on glass cover slips, maintained as previously described,[23] and transfected with plasmids encoding $GABA_A$ receptor subunit mixtures (1α:1β:2γ) using lipofectamine (Invitrogen). A eukaryotic green fluorescent protein expression plasmid, pmaxGFP (Amaxa, Gaithersburg, MD), was mixed with the $GABA_A$ receptor subunit plasmids to aid in identification of transfected cells. Transfected cells were maintained in culture medium for 24–48 h before electrophysiology experiments.

Oocyte Electrophysiology

$GABA_A$ receptor responses to GABA were assessed in *Xenopus* oocytes using two-microelectrode voltage clamp electrophysiology, as previously described.[24] GABA pulses were from 5 to 20 s, depending on the concentration of GABA used and the time to steady-state peak current. Normalizing GABA responses were recorded at maximal GABA (1-10 mM). Picrotoxin-sensitive leak currents were measured by superfusion with 2 mM picrotoxin, followed by washout for at least 5 min before testing maximal GABA response. Alphaxalone (2 μM) was used as a gating enhancer in combination with 10 mM GABA to provide estimates of GABA efficacy. Oocyte currents were low-pass filtered at 1 kHz (Model OC-725B, Warner Instruments, Hamden, CT) and digitized at 1–2 kHz using commercial digitizer hardware (Digidata 1200; Molecular Devices, Sunnyvale, CA) and software (pClamp 7; Molecular Devices).

Electrophysiology in HEK293 Cell Membrane Patches

Excised outside-out membrane patches were voltage-clamped at –50 mV, and current recordings were performed at room temperature (21–23°C) as previously described.[23] Bath and superfusion solutions contained (in mM) 145 NaCl, 5 KCl, 10 HEPES, 2 $CaCl_2$, and 1 $MgCl_2$ at pH 7.4 (pH adjusted with *N*-methyl glucosamine). The intracellular (pipette) fluid contained (in mM) 140 KCl, 10 HEPES, 1 EGTA, and 2 $MgCl_2$ at pH 7.3 (pH adjusted with KOH). Currents were stimulated with brief (0.5–1.0 s) pulses of GABA delivered *via* a multichannel superfusion pipette coupled to piezo-electric elements that switched superfusion solutions in under 1 ms. Currents were filtered at 5 kHz and digitized at 10 kHz for offline analysis.

Data Analysis

Leak-correction and measurement of peak currents were performed offline using Clampfit 8.0 software (Molecular Devices). Peak GABA-activated or etomidate-activated oocyte currents were normalized to maximal GABA-activated currents (I_{max}^{GABA}) measured in the same cell. Concentration-response curves were assembled

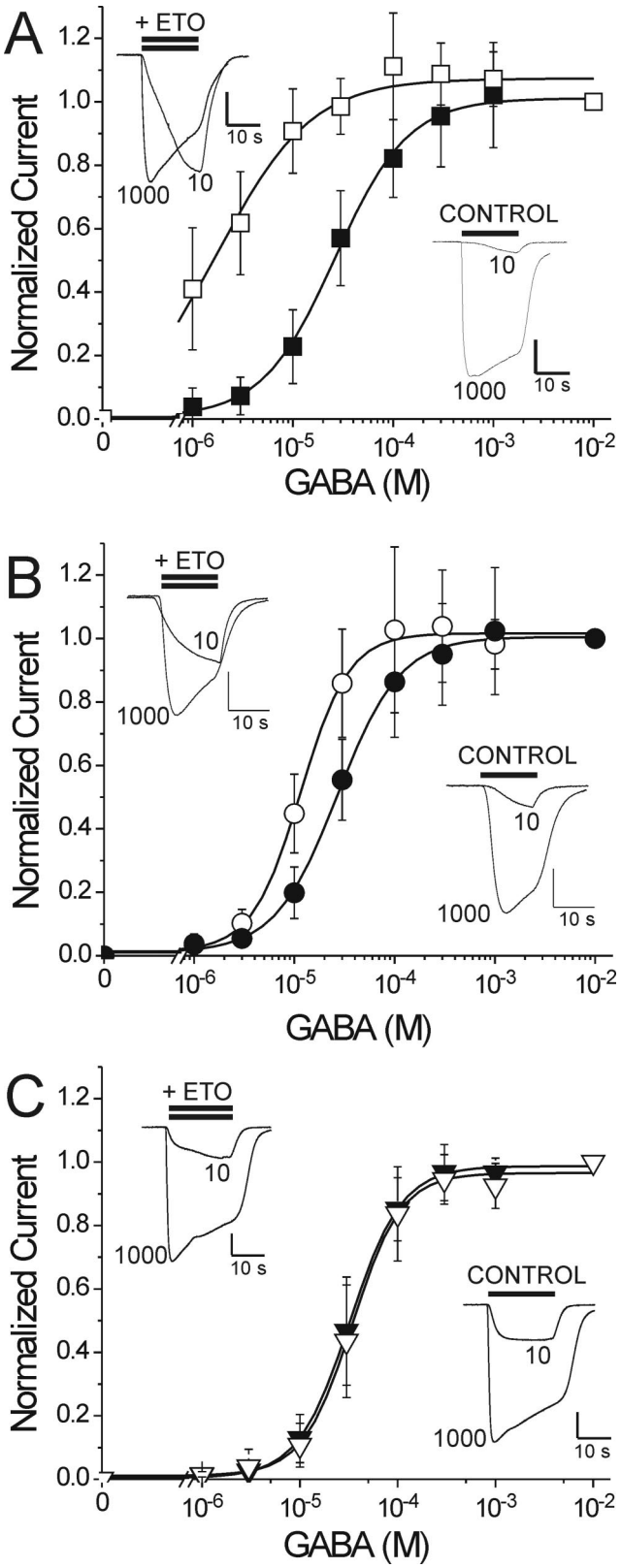

Fig. 1. γ-Aminobutyric acid (GABA) concentration-response curves and etomidate left shifts. Each *panel* displays mean ± SD data for peak GABA-activated oocyte currents normalized to the maximum response. *Solid symbols* represent control data (no etomidate). *Open symbols* represent responses measured in the presence of 3.2 μM etomidate. *Lines* through data represent nonlinear least squares fits of data to logistic functions. Fitted parameters are reported in table 1. *Lower-right insets* in each panel display examples of current sweeps elicited with either 10 or 1,000 μM GABA in a single oocyte (*bars* above sweeps indicate application). *Upper left insets* in each panel display examples of current sweeps recorded from the same oocyte in the presence of etomidate. (*A*) Wild-type α1β2γ2L (*squares*). Etomidate strongly potentiates currents at 10 μM GABA and induces a large leftward shift in GABA concentration-responses (14-fold reduction in EC_{50}). (*B*) α1β2(N265S)γ2L (*circles*). Etomidate weakly potentiates currents elicited with 10 μM GABA and induces a small leftward shift in GABA concentration-response (2.3-fold reduction in EC_{50}). (*C*) α1β2(N265M)γ2L (*triangles*). Etomidate does not enhance GABA-activated currents and does not significantly shift the GABA concentration-response curve.

from pooled normalized data from multiple oocytes. Pooled data sets were fitted with logistic (Hill) functions using nonlinear least squares in Origin 6.1 (OriginLab, Northampton, MA) and Prism 5.02, (GraphPad Software, San Diego, CA):

$$\frac{I}{I_{max}^{GABA}} = A \times \frac{[Agonist]^{nH}}{[Agonist]^{nH} + EC_{50}^{nH}} \quad (1)$$

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

Etomidate potentiation of GABA responses was quantified as the ratio of the GABA EC_{50} values in the absence of anesthetic to that in the presence of 3.2 μM etomidate. GABA concentration-response curves shift leftward (*i.e.*, to a lower GABA EC_{50}) in the presence of etomidate; thus large EC_{50} ratios indicate strong modulation, whereas a ratio of 1.0 or less indicates lack of positive modulation.[25]

Picrotoxin-sensitive leak currents (I_{PTX}) were normalized to I_{max}^{GABA}, providing estimates of basal open probability (P_0). Incorporation of the α1(L264T) mutation was used to enhance basal gating for comparison of the effects of β2N265 mutations. Maximal GABA efficacy was assessed by first activating oocyte-expressed channels with 10 mM GABA. After full current activation and partial desensitization, superfusate was switched to 10 mM GABA plus 2 μM alphaxalone, a strong positive modulator of wild-type and both mutant receptors. Maximal GABA efficacy was calculated as the ratio of current immediately before the addition of alphaxalone (I_{max}^{GABA}) to the secondary current peak after the addition of alphaxalone ($I^{GABA+alphax}$).

Estimated open probability (P_{open}^{est}), defined as the fraction of activatable receptors in the open state, was calculated by explicitly adding spontaneous current and renormalizing to the full range of open probability, assuming that picrotoxin-blocked leak represents no activation ($P_{open} = 0$) and that maximal GABA plus alphaxalone activates all nondesensitized channels ($P_{open} = 1.0$).[26] In

Table 1. β2N265 Mutation Impact on GABA Responses, Etomidate Modulation, and Direct Etomidate Activation of $GABA_A$ Receptors

		Receptor		
	Fitted Parameter‡	α1β2γ2L	α1β2(N265S)γ2L	α1β2(N265M)γ2L
GABA responses (0 Etomidate)				
	GABA EC_{50}, μM	26 ± 1.8	27 ± 1.5	32 ± 0.9*
	GABA Hill slope	1.2 ± 0.18	1.4 ± 0.15	1.6 ± 0.07*
Etomidate modulation of GABA responses				
	GABA EC_{50} with 3.2 μM etomidate, μM	1.9 ± 0.36	12 ± 0.71†	34 ± 2.1†
	EC_{50} ratio (ctl/Eto)§	14	2.3 †	0.95†
Etomidate direct activation (0 GABA)				
	Etomidate EC_{50},μM	36 ± 1.1	78 ± 25	NA
	Etomidate Hill slope	1.3 ± 0.54	1.7 ± 0.48	NA
	Etomidate efficacy (fraction GABA max)	0.43 ± 0.17	0.03 ± 0.011†	< 0.001†

Errors are calculated in Origin 6.1 (OriginLab, Northampton, MA). *P* values for fit comparisons were assessed using GraphPad Prism 5.02 (GraphPad Software, San Diego, CA). Data and fitted logistic functions are graphically displayed in figures 1 and 2D.

* Differs from wild-type at $P < 0.05$; † differs from wild-type at $P < 0.005$; ‡ fitted parameters from logistic fits to γ-aminobutyric acid (GABA) and etomidate concentration-response studies (figs. 1 and 2); § the EC_{50} ratio is the ratio of GABA EC_{50} in control experiments divided by the GABA EC_{50} in the presence of 3.2 μM etomidate.

NA = not applicable.

practice, the elements used to calculate P_{open}^{est} are all normalized to I_{max}^{GABA}:

$$P_{open}^{est} = \frac{I + I_{PTX}}{I^{GABA+Alphax} + I_{PTX}} = \frac{\frac{I}{I_{max}^{GABA}} + \frac{I_{PTX}}{I_{max}^{GABA}}}{\frac{I^{GABA+Alphax}}{I_{max}^{GABA}} + \frac{I_{PTX}}{I_{max}^{GABA}}} \quad (2)$$

Quantitative analysis based on Monod-Wyman-Changeux coagonism[5] was performed as follows: average P_{open}^{est} values calculated from GABA concentration-responses (with and without etomidate) and etomidate direct activation data were pooled. With both [GABA] and [etomidate] specified as independent variables, these data were globally fitted to equation 3 using nonlinear least squares:

$$P_{open} = \frac{1}{1 + L_0\left(\frac{1+[GABA]/K_G}{1+[GABA]/cK_G}\right)^2\left(\frac{1+[ETO]/K_E}{1+[ETO]/dK_E}\right)^2} \quad (3)$$

This equation describes a two-state equilibrium allosteric mechanism with two classes of agonist sites (one for GABA and one for etomidate), each with two equivalent sites. L_0 in equation 3 is a dimensionless basal equilibrium gating variable, approximately P_0^{-1}. K_G and K_E are equilibrium dissociation constants for GABA and etomidate binding to inactive states, and c and d are dimensionless parameters representing the respective ratios of binding constants in active *versus* inactive states. The agonist efficacies of GABA and etomidate are inversely related to c and d, respectively.

To analyze membrane patch macrocurrents for activation, desensitization, and deactivation kinetics, data windows were specified in each trace for different phases of the waveform. Activation windows were from 10% above the baseline trace to a point where desensitization had reduced the peak current by 3–5%. Desensitization windows were from the current peak to the end of GABA application. Deactivation windows were from the end of GABA application to the end of the sweep. Windowed data were fitted to multiple exponential functions using nonlinear least squares:

$$I(t) = A_1 \times \exp(-t/\tau_1) + A_2 \times \exp(-t/\tau_2) + A_3 \times \exp(-t/\tau_3) + C \quad (4)$$

The number of components for each fit was determined by comparison of single-, double-, and triple-exponential fits using an F test to choose the best exponential fit model with a confidence value of $P = 0.99$ (Clampfit8.0; Molecular Devices).

Statistical Analysis

Results are reported as mean ± SD unless otherwise indicated. Nonlinear regression errors are those from fits in Origin 6.1 (OriginLab). Statistical comparison of fitted parameters was performed using Prism 5.02 (GraphPad Software). Single parameter group comparisons were performed using either a two-tailed Student *t* test (with independent variances) or ANOVA with Tukey *post hoc* multiple comparisons test in Microsft Excel (Microsoft Corporation, Redmond, WA) with an add-on statistical toolkit (StatistiXL; Nedlands, Australia). Statistical significance was inferred at $P < 0.05$.

Results

GABA Concentration-response Relationships and Etomidate Modulation

GABA concentration-responses with and without etomidate were measured in *Xenopus* oocytes and normal-

ized to I_{max}^{GABA} without etomidate (fig. 1, table 1). A logistic fit to pooled wild-type $\alpha1\beta2\gamma2L$ receptor data revealed a GABA EC_{50} of 26 μM and a Hill slope of 1.2. Addition of 3.2 μM etomidate dramatically enhanced currents elicited with low GABA, causing a 14-fold decrease in the wild-type GABA EC_{50} to 1.9 μM and a small increase in the maximal GABA-activated current. Currents from $\alpha1\beta2$(N265S)$\gamma2L$ receptors expressed in oocytes were characterized by GABA EC_{50} = 27 μM, which was not significantly different from that of wild-type (P = 0.67). Addition of etomidate weakly enhanced currents elicited with low GABA and produced a 2.3-fold reduction in GABA EC_{50} to 12 μM. This shift was significantly smaller than that observed in wild-type ($P < 0.0001$). GABA-activated currents from $\alpha1\beta2$(N265M)$\gamma2L$ receptors were characterized by an EC_{50} value of 32 μM, significantly different from wild-type (P = 0.011). Etomidate did not enhance GABA-activated currents from this mutant, and GABA EC_{50} in the presence of 3.2 μM etomidate was 34 μM, not significantly different from GABA EC_{50} without etomidate (P = 0.64).

Etomidate Direct Activation

Etomidate, in the absence of GABA, directly activated chloride currents in oocytes expressing wild-type $\alpha1\beta2\gamma2L$ $GABA_A$ receptors (fig. 2, table 1). Maximal direct activation averaged 43% of the maximal GABA-activated current (I_{max}^{GABA}) and was observed at 0.3 mM etomidate. Half-maximal direct activation was at 36 μM etomidate. In $\alpha1\beta2$(N265S)$\gamma2L$ receptors, etomidate elicited small currents with maximum amplitude only 3% of that elicited by high GABA. Half-maximal direct activation in $\alpha1\beta2$(N265S)$\gamma2L$ receptors was at about 80 μM etomidate, but it was not significantly different from wild-type because of large uncertainty (P = 0.24). Etomidate at up to 1 mM did not elicit any currents in oocytes expressing $\alpha1\beta2$(N265M)$\gamma2L$ receptors.

Maximal GABA Efficacy

Maximal GABA efficacy was estimated by first activating receptors with a saturating concentration of GABA (10 mM, approximately 380 × EC_{50}), then adding 2 μM alphaxalone (fig. 3). Alphaxalone more than doubled currents elicited with GABA concentrations below EC_{50}, showing that it is a strong positive modulator of all three receptors (not shown). In oocytes expressing wild-type $\alpha1\beta2\gamma2L$ receptors, alphaxalone enhanced maximal GABA currents on average by 9 ± 3.6% (n = 8). Alphaxalone enhanced maximal currents in receptors containing $\beta2$(N265S) and $\beta2$(N265M) mutations by 7 ± 3.2% (n = 10) and 19 ± 6.1% (n = 7), respectively. Assuming that 10 mM GABA plus alphaxalone activates all nondesensitized channels, we infer that GABA alone has an efficacy of 0.92 ± 0.030 in wild-type, 0.93 ± 0.030 in $\alpha1\beta2$(N265S)$\gamma2L$, and 0.84 ± 0.043 in $\alpha1\beta2$(N265M)$\gamma2L$. The results for the $\beta2$(N265M) mutant demonstrate

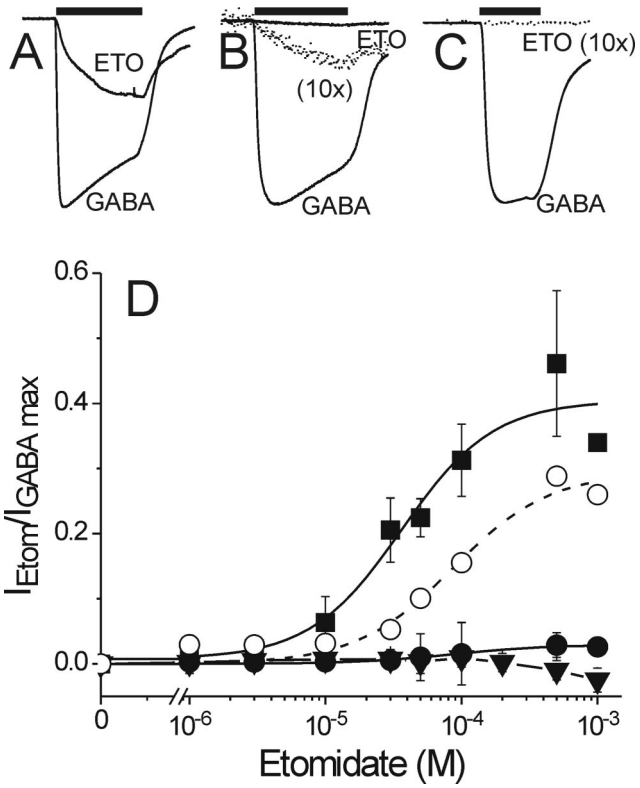

Fig. 2. Direct activation of γ-aminobutyric acid receptor type A ($GABA_A$) receptor currents by etomidate (ETO, Etom). (*A–C*) Voltage-clamp currents elicited first with 300 μM etomidate and then with 1,000 μM GABA in oocytes. Small etomidate responses are magnified ten times (*dotted sweeps*). (*A*) Wild-type $\alpha1\beta2\gamma2L$ receptors. (*B*) $\alpha1\beta2$(N265S)$\gamma2L$. (*C*) $\alpha1\beta2$(N265M)$\gamma2L$. (*D*) Mean (± SD) data from at least 5 oocytes for each type of receptor. Etomidate-activated currents are normalized to maximal GABA (1–3 mM) currents measured in the same oocyte. Wild-type (*solid squares*); $\alpha1\beta2$(N265S)$\gamma2L$ (*solid circles*); $\alpha1\beta2$(N265S)$\gamma2L$ 10 x responses (*open circles*); $\alpha1\beta2$(N265M)$\gamma2L$ (*solid triangles*). Lines through wild-type and $\beta2$(N265S) data represent nonlinear least squares fits to logistic functions. Fitted parameters are reported in table 1.

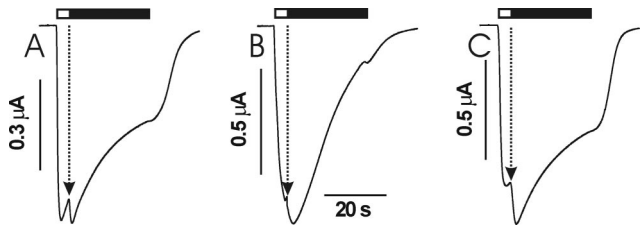

Fig. 3. The impact of $\beta2$N265 mutations on maximal γ-aminobutyric acid (GABA) efficacy. Each *panel* displays a voltage-clamp current sweep from an oocyte expressing different types of GABA receptor type A ($GABA_A$) receptors. Currents were elicited first with 10 mM GABA for 4 s (*open bar*), then with 10 mM GABA plus 2 μM alphaxalone (*solid bar*). Maximal GABA currents were enhanced by alphaxalone in all three receptors. The arrows point to the GABA current immediately before alphaxalone enhancement, which was used to calculate GABA efficacy. (*A*) Wild-type $\alpha1\beta2\gamma2L$; (*B*) $\alpha1\beta2$(N265S)$\gamma2L$; (*C*) $\alpha1\beta2$(N265M)$\gamma2L$. Alphaxalone enhances $\alpha1\beta2$(N265M)$\gamma2L$ more than wild-type or $\alpha1\beta2$(N265S)$\gamma2L$ receptors, indicating that GABA efficacy is lower in $\alpha1\beta2$(26 5M)$\gamma2L$ than in the other channels.

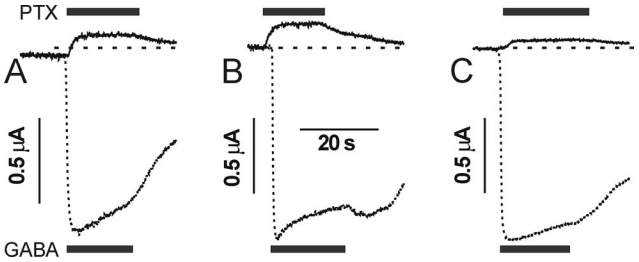

Fig. 4. Estimating the influence of β2N265 mutations on basal channel gating. A mutation in the α1 subunit (L264T) was used to produce γ-aminobutyric acid receptor type A ($GABA_A$) channels that open in the absence of GABA. A high concentration of picrotoxin (PTX; *bars above sweeps* indicate application) was applied to block spontaneously open channels, producing an apparent outward current, which was normalized to maximal GABA-activated current (GABA; *bars below sweeps* indicate application) for each type of receptor. (*A*) Wild-type α1(L264T)β2γ2L; (*B*) α1(L264T)β2(N265S)γ2L; (*C*) α1(L264T)β2(N265M)γ2L. I_{PTX}/I_{GABA} is smaller for α1(L264T)β2(N265M)γ2L than for α1(L264T)β2γ2L or α1(L264T)β2(N265S)γ2L receptors.

significantly lower GABA efficacy (ANOVA $P < 0.001$) than either of the other two receptors. Wild-type and β2(N265S) mutant results were not significantly different ($P = 0.74$).

Spontaneous Channel Activity

Basal gating of all three receptors was assessed using picrotoxin, a potent $GABA_A$ receptor inhibitor. Picrotoxin did not significantly reduce resting oocyte leak currents in cells expressing any of the three receptors ($n \geq 3$; data not shown). Given that maximal GABA currents in oocytes were as high as 7 μA and our equipment can detect changes as small as 5 nA, we infer that the basal open probability of all three receptors is less than 0.1%. To more precisely quantify the effects of mutations on basal gating, we substituted wild-type α1 subunits with α1(L264T), containing a mutation that confers measurable basal gating activity (fig. 4). Picrotoxin (2 mM) produced an apparent outward current in α1(L264T)β2γ2L receptors that averaged 12 ± 4.5% (n = 7) of maximal GABA response. In oocytes expressing α1(L264T)β2(N265S)γ2L and α1(L264T)β2(N265M)γ2L receptors, picrotoxin produced apparent outward currents that averaged, respectively, 15 ± 7.0% (n = 11) and 7 ± 2.4% (n = 7) of maximal GABA currents. The α1(L264T)β2γ2L and α1(L264T)β2(N265S)γ2L results were not significantly different (ANOVA $P = 0.15$), whereas α1(L264T)β2(N265M)γ2L results differed significantly from the other two receptors ($P = 0.0004$ *vs.* wt and $P < 0.0001$ *vs.* N265M). These results indicate that the β2(N265S) mutation causes little or no change in basal gating, while the β2(N265M) mutation reduces basal gating probability approximately two-fold.

Allosteric Coagonist Modeling

Global mechanism-based analyses of oocyte data were performed as previously described.[26] An initial fit with all parameters free to vary was used to determine the L_0 value for wild-type (25,000 ± 9,000). Our experimental results indicated that the β2(N265S) mutation does not alter basal gating; therefore, the L_0 value was constrained to equal the wild-type value in fitting α1β2(N265S)γ2L receptor data (table 2). Our data indicated that the β2(N265M) mutation reduced basal gating probability two-fold relative to wild-type channels; therefore, the L_0 value for fitting α1β2(N265M)γ2L data was fixed at twice that used for wild-type. Fits to calculated P_{open}^{est} data sets for both wild-type and α1β2(N265S)γ2L converged for all four remaining free parameters in equation 3 (Methods). α1β2(N265M)γ2L receptors are totally insensitive to etomidate; therefore, fits to P_{open}^{est} data for this receptor did not converge on values for either K_E or d. These parameters were removed from equation 3 for subsequent fit to α1β2(N265M)γ2L data, resulting in well-determined parameters for both K_G and c. Results of the nonlinear least squares fits are reported in table 2 and displayed in figure 5.

Table 2. Allosteric Coagonist Models for Wild-type and β2N265 Mutant γ-aminobutyric acid receptor type A ($GABA_A$) Receptors

	Receptor		
Model Parameter†	α1β2γ2L	α1β2(N265S)γ2L	α1β2(N265M)γ2L
L_0 (basal gating)‡	25,000	25,000	50,000
K_G (GABA dissociation constant), μM	70 ± 22	68 ± 7.4	59 ± 3.6
c^{-1} (GABA efficacy) §	530 ± 105	560 ± 37	530 ± 28
K_E (etomidate dissociation constant), μM	40 ± 9.6	88 ± 42	NA
d^{-1} (etomidate efficacy) ‖	130 ± 12	26 ± 10.8*	NA

Errors are calculated in Origin6.1 (OriginLab, Northampton, MA). *P* values for fit comparisons were assessed using GraphPad Prism 5.02 (GraphPad Software, San Diego, CA). Data and results are graphically displayed in figure 5.

* Differs from wild-type at $P < 0.005$. † Model parameters (equation 3, Methods) were fitted to estimated P_{open} data (equation 2, Methods) by using nonlinear least squares. ‡ L_0 values were fixed in the fits. The L_0 value for fits to both wild-type and α1β2(N265S)γ2L data was based on analysis of wild-type data where L_0 was allowed to vary (L_0 = 25,000 ± 9,000). For fits to α1β2(N265M)γ2L data, the value for L_0 was set at twice the value used for wild-type, and K_E and d were removed from the fitting equation. § GABA efficacy is inversely related to c. ‖ Etomidate efficacy is inversely related to d.

NA = not applicable.

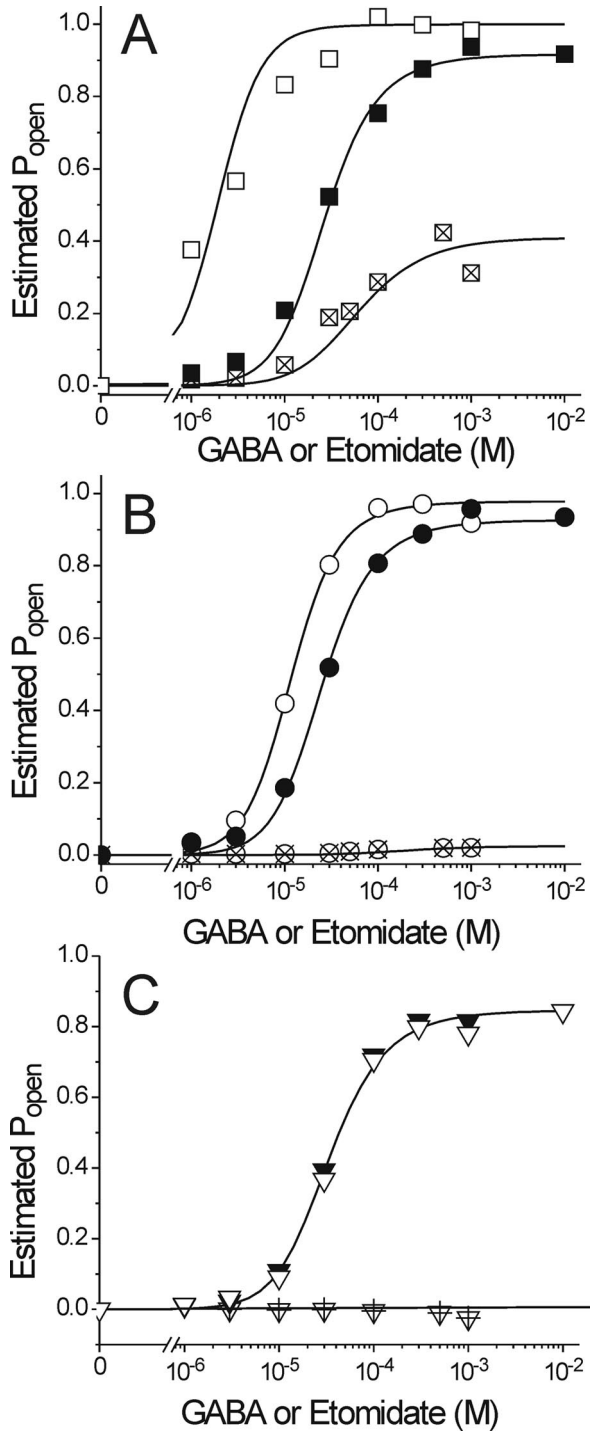

Fig. 5. Allosteric coagonist models for γ-aminobutyric acid (GABA) and etomidate-dependent receptor activation. Estimated open probabilities were calculated from mean normalized concentration-response data (figs. 1 and 2) using equation 2. *Solid symbols* represent control GABA concentration-responses, *open symbols* represent GABA responses in the presence of 3.2 μM etomidate, and *cross-hatched symbols* represent etomidate direct activation responses. Combined data for each channel type were fitted with equation 3 (Methods), and results are plotted as *solid lines* in the panels. Fitted parameters are reported in table 2. (*A*) Wild-type α1β2γ2L (*square symbols*); (*B*) α1β2(N265S)γ2L (*circles*); (*C*) α1β2(N265M)γ2L (*triangles*).

Macrocurrent Kinetics

GABA-activated currents in outside-out patches excised from transfected HEK293 cells were used to study macrocurrent kinetics of $GABA_A$ receptors (fig. 6, table 3). As previously reported,[23] α1β2γ2L wild-type currents elicited with high GABA concentrations display rapid activation, multiphasic desensitization, and biphasic deactivation after discontinuation of agonist. Currents elicited from patches expressing mutant receptors showed similar kinetics, which were analyzed in detail. For wild-type and both mutants, the best fit (F test; $P = 0.99$) number of exponential deactivation phases was two in all patches, and the best-fit number of desensitization phases was two in the majority (18 of 23) of patches. Thus, for comparison, deactivation and desensitization phases of all patches were reanalyzed by using double-exponential functions. Statistical comparison with ANOVA indicates that fitted time constants for activation, desensitization, and deactivation are indistinguishable for all three types of receptor channels (table 3). Fast deactivation rate analysis suggested that this phase in α1β2(N265M)γ2L may be faster than in wild-type receptors (59 s^{-1} *vs.* 42 s^{-1}), although this difference was not statistically significant ($P = 0.15$).

Etomidate Activation of Spontaneously Gating Receptors with β1 versus *β2*

A final set of experiments addressed the efficacy and potency of etomidate direct activation in spontaneously active receptors containing the α1(L264T) mutation combined with γ2L and either β2 or β1 subunits (fig. 7). Oocyte-expressed α1β1γ2L receptors displayed no detectable (less than 0.1%; n = 5) picrotoxin-sensitive spontaneous activity and are characterized by GABA EC_{50} = 190 μM (fig. 7A, solid diamonds). Oocyte-expressed α1(L264T)β1γ2L receptors display 11 ± 2.1% (n = 5) spontaneous activity and GABA EC_{50} = 3.8 μM (fig. 7A, open diamonds). High GABA responses are not significantly enhanced by alphaxalone in either α1(L264T)β1γ2L or α1(L264T)β2γ2L (not shown), indicating that maximal GABA efficacy in both gating mutant channels is near 1.0. Etomidate potently (EC_{50} = 0.8 μM) activates α1(L264T)β2γ2L receptors, with efficacy that is similar to GABA in these channels (fig. 7B, solid triangles). By using a value of 8 for L_0 (calculated from I_{ptx}/I_{GABA} results; fig. 4) along with K_E = 40 μM and d = 0.0076 from the wild-type α1β2γ2L global model fit (table 2), equation 3 closely predicts etomidate direct activation in α1(L264T)β2γ2L receptors. In contrast, the etomidate EC_{50} for direct activation of α1(L264T)β1γ2L receptors is 43 μM, and maximal etomidate activation reaches about 60% of the maximal GABA response. The α1(L264T)β1γ2L etomidate direct activation data were used to calculate P^{est}_{open} (equation 2, methods) and fitted with equation 3, resulting in L_0 = 9, K_E = 58 μM, and d = 0.26. Our estimated parameters for etomidate bind-

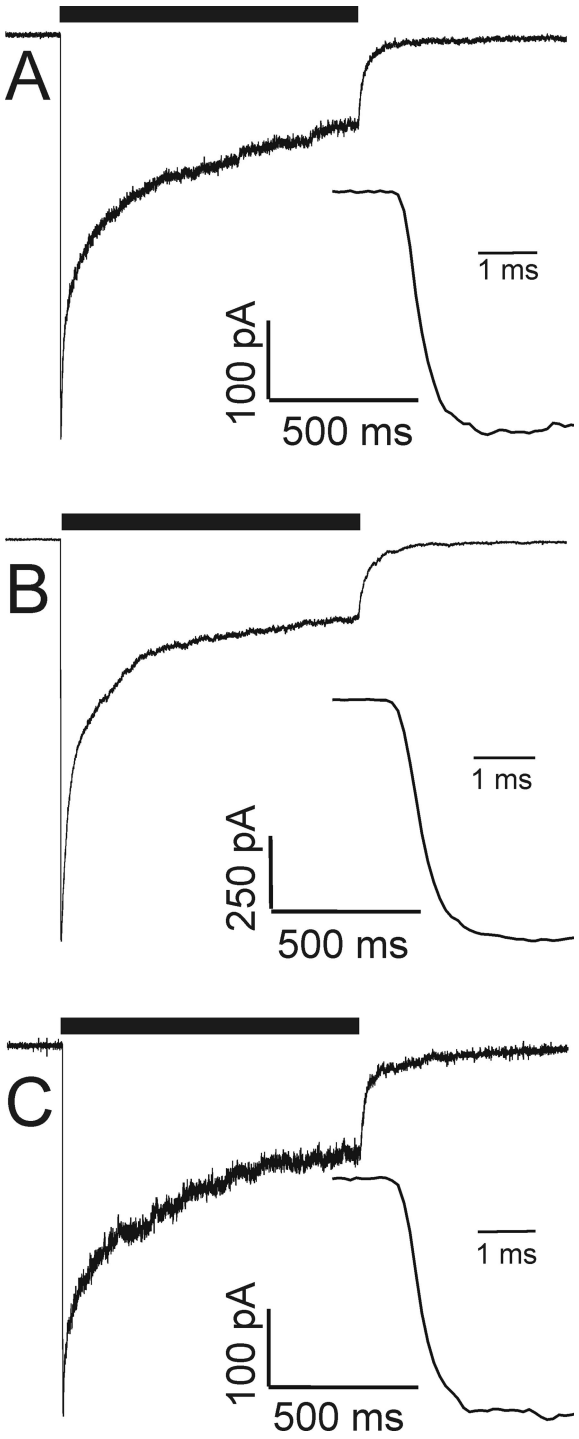

Fig. 6. γ-Aminobutyric acid receptor type A ($GABA_A$) receptor current kinetics from rapidly superfused patch-clamp studies. Each *panel* displays a single current sweep recorded from a patch exposed to 1 mM GABA for 1 s (indicated by the *bars over the current traces*). Currents display three distinct phases that were analyzed separately: *activation* is the rapid evolution of GABA-activated inward current; *desensitization* is the drop in current during GABA application; and *deactivation* is the return to baseline after termination of GABA application. *Inset sweeps* display activation phases at an expanded scale. Results of kinetic analyses are summarized in table 3. (*A*) Wild-type α1β2γ2L; (*B*) α1β2(N265S)γ2L; (*C*) α1β2(N265M)γ2L.

ing to closed receptors containing β1 *versus* β2 subunits differ by less than 50% (K_E = 58 μM with β1 *vs.* 40 μM with β2), whereas etomidate efficacy (inversely related to d) is 34 times larger in β2-containing receptors *versus* β1-containing receptors (d^{-1} = 3.85 with β1 *vs.* 132 with β2).

Discussion

Our aim in this study was to better define the role of the β subunit residue at position 265 in $GABA_A$ receptor function in both the absence and presence of etomidate. Previous studies have reported diminished sensitivity to etomidate in heterologously expressed $GABA_A$ receptors containing either β2 or β3 subunits with N265S[15] or N265M mutations.[13,16,17,27,28] Unlike previously published reports, our experiments quantitatively assessed etomidate modulation of GABA activation as the ratio of GABA EC_{50}s in the absence and presence of a standard etomidate concentration, a robust measure of positive allosteric modulation.[25,29] This approach demonstrates that, in α1β2γ2L $GABA_A$ receptors, the β2(N265S) mutation reduces etomidate modulation by sixfold (2.3-fold shift *vs.* 14-fold for wild-type), whereas β2(N265M) eliminates this shift entirely. We further tested the impact of the β2N265 mutations on direct receptor activation by etomidate. We found that etomidate elicits more than 40% of maximal GABA current in wild-type, about 3% in α1β2(N265S)γ2L receptors, and no detectable current in α1β2(N265M)γ2L receptors. Thus, the effect of the β2N265 mutations on etomidate direct activation parallels that on modulation of GABA responses. Previous studies have also noted that both direct etomidate activation and modulation of GABA responses are reduced in receptors containing β1 *versus* β2[12] or those containing β2/3(N265M) mutations.[13]

We also evaluated whether β2N265 mutations alter $GABA_A$ receptor gating in the absence of etomidate, including experiments to measure spontaneous (0 GABA) gating activity, GABA EC_{50}, maximal GABA efficacy, and the macroscopic rates of transitions among major functional states. We found that none of these parameters was significantly altered by the β2(N265S) mutations, whereas the β2(N265M) mutation produced a 23% increase in GABA EC_{50} (table 1), and also significantly reduced maximal GABA efficacy from 0.92 to 0.84. Whereas we could detect no spontaneous gating in wild-type, α1β2(N265S)γ2L, or α1β2(N265M)γ2 channels, novel experiments exploiting the spontaneously gating α1(L264T)β2γ2L mutant channel background reveal that substituting β2(N265M) for β2 reduces basal gating activity by 50%, and substitution with β2(N265S) causes no significant change. Analysis of GABA-activated current kinetics recorded using submillisecond concentration jumps shows that activation, desensitization, and

Table 3. β2N265 Mutation Impact on GABA-stimulated Activation, Desensitization, and Deactivation Time Constants

	Receptor		
Current Phase	$\alpha1\beta2\gamma2$L	$\alpha1\beta2$(N265S)$\gamma2$L	$\alpha1\beta2$(N265M)$\gamma2$L
Activation τ, ms (n)	0.61 ± 0.34 (7)	0.72 ± 0.28 (6)	0.63 ± 0.18 (6)
Fast desensitization τ, ms	33 ± 9.7	26 ± 11	23 ± 12
Fraction fast (n)	0.4 ± 0.09 (5)	0.2 ± 0.17 (5)	0.2 ± 0.09 (5)
Slow desensitization τ, ms	1,300 ± 430	1,100 ± 180	1,300 ± 560
Fraction slow (n)	0.6 ± 0.09 (5)	0.8 ± 0.17 (5)	0.8 ± 0.09 (5)
Fast deactivation τ, ms	24 ± 7.6	21 ± 3.9	17 ± 8.7
Fraction slow (n)	0.6 ± 0.35 (7)	0.5 ± 0.30 (6)	0.5 ± 0.13 (6)
Slow deactivation τ, ms	210 ± 140	190 ± 150	140 ± 100
Fraction fast (n)	0.4 ± 0.35 (7)	0.5 ± 0.30 (6)	0.5 ± 0.13 (6)

Results are mean ± SD time constants (τ) for different phases of macrocurrents recorded by using rapid patch superfusion (n indicates number of patches analyzed). The activation phase (current growth during γ-aminobutyric acid [GABA] application) was fitted to single exponential functions by using nonlinear least squares regression. Desensitization (current decline during GABA application) and deactivation (current decline after GABA washout) phases were fitted to double exponential functions. Statistical comparisons performed by using ANOVA identified no differences that were significant at $P < 0.05$.

deactivation rates are not significantly altered by either β2(N265S) or β2(N265M), although rapid deactivation tended to be faster in $\alpha1\beta2$(N265M)$\gamma2$L receptors than in wild-type (table 3).

A number of previous studies have reported that β2(N265M) or β3(N265M) mutations increase GABA EC_{50}[16,17,28,30]. Nishikawa *et al.*[30] studied a series β2N265 mutations in $\alpha1\beta2\gamma2$L receptors in HEK293 cells and found that GABA EC_{50} approximately doubled with β2(N265M) and increased about 50% with β2(N265S). One previously published study by Miko *et al.*[31] investigated spontaneous gating in homomeric $\beta1$ $GABA_A$ receptors and the effects of β1(S265) mutations. In that study, mutant β3(N265S) subunits did not induce spontaneous gating in homomeric or heteromeric channels. No previous studies have assessed the influence of β2N265 or β3N265 mutations on macrocurrent kinetics in heterologously expressed channels. However, because GABA reuptake from synapses occurs in less than a millisecond,[32] GABAergic inhibitory postsynaptic current decay is largely dependent on channel deactivation. Reynolds *et al.*[2] reported that GABAergic miniature inhibitory postsynaptic current decays were similar in cerebellar Purkinje neurons from both wild-type and β2(N265S) knock-in mice. Drexler *et al.*[33] found that inhibitory postsynaptic current decays in neocortical neurons of wild-type and β3(N265M) knock-in mice were not significantly different. The subunit compositions of channels mediating these neuronal inhibitory postsynaptic currents is uncertain, but inhibitory postsynaptic current prolongation by etomidate was dramatically reduced in currents recorded from knock-in *versus* wild-type cells, demonstrating that $GABA_A$ receptors containing mutant β subunits were present. These results therefore indicate that *in vivo* neuronal $GABA_A$ channel kinetics are minimally altered by N265S and N265M mutations.

Allosteric gating models have proven useful for interpretation of how mutations alter the function of ligand-gated ion channels.[34,35] We have used allosteric coagonist models to interpret both etomidate actions on $GABA_A$ receptors[5] and the effects of mutations on etomidate sensitivity,[26] and we have applied this approach here to analyze the impact of the β2N265 mutants we studied (fig. 5, table 2). Importantly, allosteric coagonist models appear to fit our wild-type and mutant data well, reinforcing the hypothesis that both etomidate modulation of GABA activation and direct etomidate activation of $GABA_A$ receptors are manifestations of interactions at a single class of etomidate sites observed under different experimental conditions.[5] After correcting L_0 values according to our basal gating results, GABA binding (K_G) and efficacy (c^{-1}) parameters in all three fitted models (table 2) are all quite similar and not statistically different, suggesting that these mutations have little or no impact on GABA interactions. The total lack of etomidate effects in $\alpha1\beta2$(N265M)$\gamma2$L receptors makes it impossible to fit etomidate binding and efficacy parameters in the allosteric model. However, comparison of coagonist model parameters for wild-type *versus* $\alpha1\beta2$(N265S)$\gamma2$L receptors indicates that the β2(N265S) mutation reduces etomidate efficacy (d^{-1}) five-fold from wild-type, whereas etomidate binding ($1/K_E$) affinity is reduced about two-fold.

Direct activation experiments using the gating mutant α1(L264T) expressed together with $\beta1$ *versus* $\beta2$ also addressed whether etomidate binding *versus* efficacy is affected by changing β subunit subtype. Combining the gating mutant L_0 with fitted values for etomidate binding (K_E) and efficacy (d) from the $\alpha1\beta2\gamma2$L model accurately predicts etomidate direct activation of α1(L264T)$\beta2\gamma2$L channels. This result suggests that the α1(L264T) mutation does not affect etomidate binding or efficacy, but simply sensitizes receptors to etomidate gating by increasing their tendency to open. Most importantly, α1(L264T)$\beta1\gamma2$L and α1(L264T)$\beta2\gamma2$L channels display similar spontaneous activity, but those with $\beta1$ subunits display less etomidate direct activation and much lower

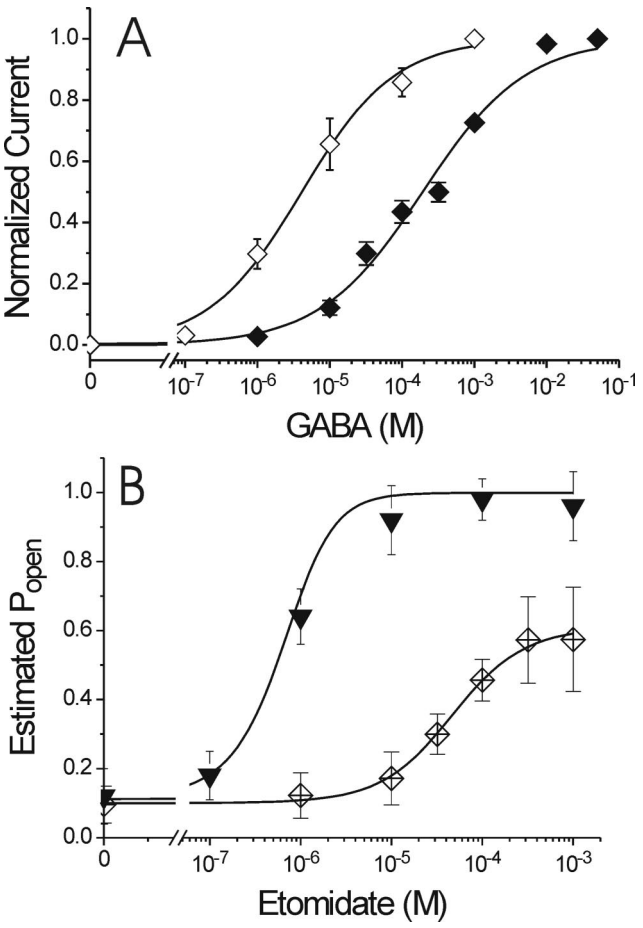

Fig. 7. The α1(L264T) gating mutation used to interpret etomidate interactions with γ-aminobutyric acid receptor type A ($GABA_A$) receptors containing β1 or β2 subunits. *Panel A* depicts GABA concentration-response data (mean ± SD) for oocytes expressing α1β1γ2L (*solid diamonds*) and α1(L264T)β1γ2L receptors (*open diamonds*). Lines represent fits to logistic functions: α1β1γ2L GABA EC_{50} = 190 ± 32 μM, Hill slope = 0.61 ± 0.064; α1(L264T)β1γ2L GABA EC_{50} = 3.8 ± 0.43 μM, Hill slope = 0.55 ± 0.073. (*B*) Estimated P_{open} values (mean ± SD) from etomidate concentration-response studies of both α1(L264T)β1γ2L (*crossed diamonds*) and α1(L264T)β2γ2L (*solid triangles*). *Lines* through α1(L264T)β1γ2L points represent a nonlinear least squares fit to equation 3 with [GABA] = 0: L_0 = 9.1 ± 0.84, K_E = 58 ± 6.4 μM, d = 0.26 ± 0.038. The *line* through α1(L264T)β2γ2L data are equation 3 with L_0 = 8 and other values set at those derived for α1β2γ2L receptors(K_E = 40 μM, d = 0.0076).

apparent affinity (etomidate EC_{50}) than those with β2 subunits. Allosteric mechanism analysis indicates that these changes are largely due to etomidate efficacy (d^{-1}) that is 34-fold lower in α1(L264T)β1γ2L *versus* α1(L264T)β2γ2L.

The analysis of β2N265 mutants contrasts with our recent study of tryptophan mutations at α1M236 and β2M286,[26] and it is most consistent with the conclusion that β2N265 is not a contact point for etomidate binding to $GABA_A$ receptors. Instead this residue may act as a transduction element between the etomidate sites and the ion channel. Other evidence also supports the conclusion that β2N265 does not contribute to etomidate binding. Recent structural models of the $GABA_A$ receptor transmembrane domains,[11,18,19] based on disulfide crosslinking and photolabeling data, depict βN265 located outside the intersubunit cleft where both etomidate and propofol bind. Furthermore, propofol does not protect cysteine substitutions at β2N265 from covalent modification, whereas it does protect cysteine modification at β2M286,[36] a site where there is abundant evidence for contact with etomidate.[11,26] An alternative interpretation of our findings is based on the concept of efficacy in allosteric gating models. Agonist (or coagonist) efficacy is the ratio of equilibrium binding dissociation constants in the two canonical states: active (open channel) *versus* inactive (closed channel). Thus, reduced etomidate efficacy in β2N265 mutants implies reduced etomidate affinity for the transient open $GABA_A$ receptor state. This introduces the possibility that, although β2N265 does not contribute to etomidate binding to inactive $GABA_A$ receptors, it might make contact with etomidate during channel opening. This interpretation also points out the importance of defining anesthetic binding determinants in more than one receptor state, a task that may be achieved using time-resolved photolabeling.[37,38]

Mutations that alter $GABA_A$ receptor anesthetic sensitivity *in vitro* represent potential tools for knock-in animal studies linking subunits to the various actions of anesthetics.[39] In this regard, the β2(N265S) mutation is remarkable for reducing etomidate sensitivity without affecting basal or GABA-activated receptor activity. Indeed, Reynolds *et al.*[2] reported that β2(N265S) knock-in mice have normal receptor expression, normal baseline and sleeping electroencephalographic activity, and normal baseline behavior. In comparison with β2(N265S), the β2(N265M) mutation produces two molecular effects: a small negative gating effect in the absence of etomidate combined with abolition of etomidate sensitivity. However, whereas the viability and general behavior of β3(N265M) knock-in mice is apparently normal, no data have been published on their awake or sleeping electroencephalographic patterns or susceptibility to seizures. One behavioral study[40] reported that baseline freezing in response to a learned fear context was significantly lower in β3(N265M) knock-ins *versus* wild-type mice.

$GABA_A$ receptor mutations with negative gating effects *in vitro*, such as γ2(K289M), are associated with increased GABA EC_{50} and with epilepsy *in vivo*,[41] whereas gain-of-function mutations such as α1(S270H) reduce GABA EC_{50} and have been associated with grossly abnormal anatomic and behavioral phenotypes in knock-in animals.[42] Allosteric models illustrate that *in vitro* GABA EC_{50} is a function of baseline channel open probability (L_0), GABA-binding affinity (K_G), and efficacy (c). A single mutation could simultaneously alter both L_0 and

GABA efficacy, resulting in a near-normal GABA EC_{50}, but significantly abnormal channel activity. Therefore, GABA EC_{50} alone may be a misleading predictor of channel activity and *in vivo* phenotype. Assessment of basal gating changes and maximal GABA efficacy, together with GABA EC_{50}, will provide a stronger basis for this prediction.

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