Competitive Antagonism of Anesthetic Action at the γ-Aminobutyric Acid Type A Receptor by a Novel Etomidate Analog with Low Intrinsic Efficacy

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ABSTRACT

Background: The authors characterized the γ-aminobutyric acid type A receptor pharmacology of the novel etomidate analog naphthalene–etomidate, a potential lead compound for the development of anesthetic-selective competitive antagonists.

Methods: The positive modulatory potencies and efficacies of etomidate and naphthalene–etomidate were defined in oocyte-expressed $\alpha_1\beta_3\gamma_{2L}$ γ-aminobutyric acid type A receptors using voltage clamp electrophysiology. Using the same technique, the ability of naphthalene–etomidate to reduce currents evoked by γ-aminobutyric acid alone or γ-aminobutyric acid potentiated by etomidate, propofol, pentobarbital, and diazepam was quantified. The binding affinity of naphthalene–etomidate to the transmembrane anesthetic binding sites of the γ-aminobutyric acid type A receptor was determined from its ability to inhibit receptor photoaffinity labeling by the site-selective photolabels [³H]azi-etomidate and R-[³H]5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid.

Results: In contrast to etomidate, naphthalene–etomidate only weakly potentiated γ-aminobutyric acid–evoked currents and induced little direct activation even at a near-saturating aqueous concentration. It inhibited labeling of γ-aminobutyric acid type A receptors by [³H]azi-etomidate and R-[³H]5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid with similar half-maximal inhibitory concentrations of 48 μM (95% CI, 28 to 81 μM) and 33 μM (95% CI, 20 to 54 μM). It also reduced the positive modulatory actions of anesthetics (propofol > etomidate ~ pentobarbital) but not those of γ-aminobutyric acid or diazepam. At 300 μM, naphthalene–etomidate increased the half-maximal potentiating propofol concentration from 6.0 μM (95% CI, 4.4 to 8.0 μM) to 36 μM (95% CI, 17 to 78 μM) without affecting the maximal response obtained at high propofol concentrations.

Conclusions: Naphthalene–etomidate is a very low-efficacy etomidate analog that exhibits the pharmacology of an anesthetic competitive antagonist at the γ-aminobutyric acid type A receptor. **(ANESTHESIOLOGY 2017; 127:824-37)**

ANESTHESIOLOGISTS often use competitive antagonists to pharmacologically reverse the actions of therapeutic drugs including opioids, muscle relaxants, benzodiazepines, and anticoagulants.[1–6] Commonly, competitive antagonists are structurally similar to the therapeutic drugs they reverse (*i.e.*, they are analogs) and bind to the same protein-binding site(s). However, they possess very little or no intrinsic efficacy for altering the function of the target protein. They are highly valuable in clinical medicine because they allow the magnitude and duration of drug effects to be precisely controlled. They are also useful in scientific research as pharmacologic tools to define the role that specific protein targets play in producing particular *in vitro* or *in vivo* drug effects.[7–10] Unfortunately, competitive antagonists for general anesthetic agents have not yet been developed, and the long-held (but now widely discredited) view that general anesthetics act nonspecifically *via* lipid membranes implied that such antagonists could never be created.[11–17]

It is now generally accepted that, similar to most other therapeutic drugs, anesthetics act quite specifically by

What We Already Know about This Topic

- Our capacity to antagonize the effects of general anesthetics, unlike that of opioids and benzodiazepines, is currently limited. The identification of general anesthetic binding sites in the γ-aminobutyric acid type A receptor offers the possibility of the development of specific anesthetic antagonists.
- The efficacy of naphthalene–etomidate, a novel etomidate analog, to antagonize anesthetic action was evaluated *in vitro*.

What This Article Tells Us That Is New

- Naphthalene–etomidate only weakly potentiated γ-aminobutyric acid–evoked currents. However, it significantly decreased the positive modulatory effects of etomidate, propofol, and pentobarbital at the γ-aminobutyric acid type A receptor.
- The results suggest that naphthalene–etomidate acts as a competitive antagonist of anesthetics at the γ-aminobutyric acid type A receptor.

binding to discrete sites on proteins.[18–20] In particular, the γ-aminobutyric acid type A (GABA_A) receptor is now known to be the principle target for the hypnotic actions of propofol

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and etomidate, and an important target for anesthetic barbiturates.[21–25] These drugs are highly efficacious positive allosteric modulators (PAMs) of GABA$_A$ receptor function, potentiating GABA$_A$ receptor-mediated currents that are evoked by low γ-aminobutyric acid (GABA) concentrations and directly activating GABA$_A$ receptors in the absence of GABA.[26–30]

Recently, two classes of general anesthetic binding sites have been identified by photoaffinity labeling studies within the transmembrane domain of the GABA$_A$ receptor.[31,32] One class of sites is photolabeled by [^{3}H]azi-etomidate and located at the two β^+–α^- subunit interfaces, whereas the other is photolabeled by R-[^{3}H]5-allyl-1-methyl-5-(m-trifluoromethyl-diazirynylphenyl) barbituric acid (R-[^{3}H]mTFD-MPAB) and located at the α^+–β^- and γ^+–β^- interfaces. Protection studies using these two photolabels have been used to define the selectivities of various anesthetics for these sites. They show that etomidate binds with more than 100-fold higher affinity to the β^+–α^- sites as compared to the α^+–β^-/γ^+–β^- sites, whereas pentobarbital exhibits the reverse selectivity, and propofol exhibits essentially no selectivity at all.[32] Photoaffinity labeling has also established that these transmembrane-binding sites for general anesthetics (PAMs) can also bind negative allosteric modulators that are GABA$_A$ receptor inhibitors.[33] These transmembrane anesthetic sites are distinct from those that bind GABA and benzodiazepines as the latter, while also located between subunits, are found within the extracellular domain.[34,35] This highly specific receptor mechanism suggests the possibility of developing anesthetic analogs that bind selectively to the transmembrane anesthetic binding sites but possess little or no intrinsic efficacy for positively modulating GABA$_A$ receptor function. We hypothesized that such analogs would act as competitive anesthetic antagonists capable of selectively reversing the GABA$_A$ receptor actions of more efficacious anesthetic agents. In this manuscript, we describe the GABA$_A$ receptor pharmacology of naphthalene–etomidate, a novel etomidate analog with very low intrinsic efficacy that selectively antagonizes anesthetic action.

Materials and Methods

Anesthetics and Anesthetic Photoaffinity Labels
Figure 1 shows the molecular structures of etomidate and naphthalene–etomidate. Etomidate was purchased from Bachem Americas (USA). Propofol, pentobarbital, and diazepam were purchased from Sigma-Aldrich (USA). Azi-etomidate and R-TFD-MPAB were synthesized as previously described.[36,37] Naphthalene–etomidate was synthesized by Aberjona Laboratories (USA).
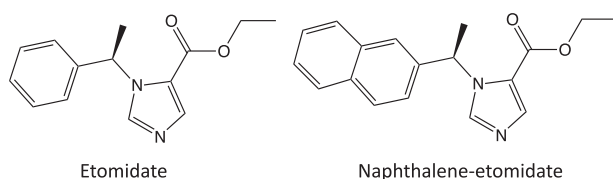

Fig. 1. Molecular structures of etomidate and naphthalene–etomidate.

GABA$_A$ Receptor Electrophysiology
Oocytes were harvested from *Xenopus* frogs with the approval of and in accordance with rules and regulations of our Institutional Animal Care and Use Committee (Massachusetts General Hospital, Boston, Massachusetts), injected with messenger RNA encoding the α_1, β_3, and γ_{2L} subunits of the human GABA$_A$ receptor, and the resulting expressed GABA$_A$ receptors were studied using the whole cell two-electrode voltage clamp technique as previously described.[38] For all studies of GABA potentiation, a GABA concentration-peak current response curve was generated for each oocyte to define the GABA concentration that elicits either 5% or 50% of the current evoked by 1 mM GABA (*i.e.*, EC$_5$ GABA or EC$_{50}$ GABA, respectively). Between electrophysiologic recordings, oocytes where perfused with buffer for at least 3 min (washout period) to remove GABA and/or drugs and to allow receptors to recover from desensitization.

Electrophysiologic Protocols to Study Modulation of GABA$_A$ Receptors by Etomidate and Naphthalene–Etomidate
Potentiation of GABA-evoked Currents. The oocyte was first perfused with 1 mM GABA, and the maximal peak current response was recorded. After a washout period, the oocyte was perfused with EC$_5$ GABA alone for 15 to 20 s followed immediately by EC$_5$ GABA plus drug (etomidate or naphthalene–etomidate) at the desired concentration for 20 to 60 s, and the peak current response was recorded. After another washout period, the oocyte was again perfused with 1 mM GABA, and the maximal peak current response was recorded. The current response recorded in the presence of EC$_5$ GABA plus drug was then normalized to the average of the two current responses evoked by 1 mM GABA.

Direct Activation of GABA$_A$ Receptors. The oocyte was first perfused with 1 mM GABA, and the maximal peak current response was recorded. After a washout period, the oocyte was perfused with the desired concentration of drug (etomidate or naphthalene–etomidate) for 15 to 20 s, and the peak current response was recorded. After another washout period, the oocyte was again perfused with 1 mM GABA, and the maximal peak current response was recorded. The peak current response in the presence of drug was then normalized to the average of the two current responses produced by 1 mM GABA.

Electrophysiology Protocols to Study Interactions between Naphthalene–Etomidate, GABA, and PAMs
To evaluate the ability of naphthalene–etomidate to modify current responses evoked by EC$_{50}$ GABA or a combination of EC$_5$ GABA plus a PAM (*i.e.*, etomidate, propofol, pentobarbital, or diazepam), we utilized three drug administration protocols.

Simultaneous Exposure Protocol. The oocyte was perfused with either (1) EC$_{50}$ GABA or (2) EC$_5$ GABA plus the desired PAM for 10 s, and the control peak current response was recorded. After a washout period, the oocyte was again perfused with EC$_{50}$ GABA or EC$_5$ GABA plus the desired

PAM but this time along with 300 µM naphthalene–etomidate, and the test peak current response was recorded. After another washout period, the control peak response obtained without naphthalene–etomidate was again recorded. The percent current amplitude change produced by naphthalene–etomidate was then defined from the difference between the peak current response recorded during the test experiment and the average of the two control peak current responses.

Naphthalene–Etomidate Preexposure Protocol. The oocyte was perfused with either (1) EC_{50} GABA or (2) EC_5 GABA plus the desired PAM for 10 s, and the control peak current response was recorded. After a washout period, the oocyte was preexposed to 300 µM naphthalene–etomidate for 10 s before coapplication with either EC_{50} GABA or EC_5 GABA plus the desired PAM for 10 s, and the test peak current response was recorded. After another washout period, the control peak response obtained without naphthalene–etomidate was again recorded. The percent current amplitude change produced by naphthalene–etomidate was then defined from the difference between the peak current response recorded during the test experiment and the average of the two control peak current responses.

GABA Preexposure Protocol. The oocyte was perfused (*i.e.*, activated) with either (1) EC_{50} GABA or (2) EC_5 GABA plus the desired PAM for 30 s. Ten seconds into this activation period, 300 µM naphthalene–etomidate was added for 10 s. The effect of naphthalene–etomidate on currents was quantified as the maximum change in current amplitude recorded during naphthalene–etomidate administration. To correct for receptor desensitization (and current run up or run down) during naphthalene–etomidate administration, an interpolated straight line was fit between the pre- and post-naphthalene–etomidate phases of the current recording period. That line was then used as the baseline against which the effect of naphthalene–etomidate was quantified. The percent current amplitude change produced by naphthalene–etomidate was then defined from the maximal current difference between that interpolated line amplitude and the recorded current amplitude at the same time point.

Photoaffinity Label Competition Experiments

$\alpha_1\beta_3\gamma_{2L}$ GABA_A receptors containing a FLAG epitope on the N terminus of the α_1 subunit were heterologously expressed in a tetracycline-inducible, stably transfected HEK 293S cell line and affinity purified on an anti-FLAG resin as previously described.[32,39] Purified receptors were then photolabeled (for 30 min) with either [³H]azi-etomidate (~2.6 µM; ~2.5 µCi/analytic sample) or R-[³H]*m*TFD-MPAB (~1.4 µM; ~2.7 µCi/analytic sample) using a 365-nm lamp in the presence of ranging concentrations of naphthalene–etomidate. Photolabel incorporation into each receptor subunit was then measured by running solubilized receptor membranes on a gel, cutting out the Coomassie Blue–stained bands corresponding to each subunit, and measuring the radioactivity in the bands as previously described.[32,40]

Data Analysis

Concentration-response curves for potentiation of EC_5 GABA-evoked currents and direct activation of GABA_A receptor currents were fit using Prism 6.0h software (GraphPad, USA) using its built-in four-parameter equation for stimulation (equation 1):

$$\text{Normalized Current Amplitude} = \text{Minimum} + \frac{\text{Maximum-Minimum}}{1+10^{(\text{LogEC}_{50}-[\text{drug}])^*n}}$$

where Minimum is the normalized peak current amplitude in the absence of drug, Maximum is the normalized peak current amplitude at high drug concentrations, [drug] is the drug concentration, EC_{50} is the drug concentration that evokes a peak current amplitude that is halfway between the maximum and minimum values, and n is the slope of the relationship. The minimum value was constrained to the experimentally determined value in the absence of drug.

Naphthalene–etomidate concentration-response curves for inhibition of 10 µM propofol potentiated currents and for inhibition of photoaffinity labeling were fit using Prism 6.0h software using its built-in four-parameter equation for inhibition (equation 2):

$$\text{Normalized Current Amplitude}$$
$$(\text{or Normalized Specific Counts per Minute})$$
$$= \text{Minimum} + \frac{\text{Maximum-Minimum}}{1+10^{([\text{NE}]-\text{LogIC}_{50})^*n}}$$

where Minimum is the normalized peak current amplitude (or normalized specific counts/min for photoaffinity labeling studies) in the presence of high naphthalene–etomidate concentrations, Maximum is the normalized peak current amplitude (or normalized specific counts/min for photoaffinity labeling studies) in the absence of naphthalene–etomidate, [NE] is the naphthalene–etomidate concentration, IC_{50} is the naphthalene–etomidate concentration that produces a current amplitude (or normalized specific counts/min for photoaffinity labeling studies) that is halfway between the maximum and minimum values, and n is the slope of the relationship. The maximum value was constrained to the experimentally determined value in the absence of naphthalene–etomidate.

Allosteric Modeling of Direct Activation Data

Allosteric receptor modeling was used to derive the microscopic dissociation constants for etomidate and naphthalene–etomidate to the open and closed conformational states of the GABA_A receptor. Etomidate and naphthalene–etomidate direct activation data were transformed to P_{open} values by assuming that P_{open} in the presence of a maximally activating GABA concentration (1 mM) is approximately 0.85.[41,42] The relationship between P_{open} and the drug concentration was then fit to the allosteric equation (equation 3):[41]

$$P_{open} = \frac{1}{1+L_0\left(\dfrac{1+[\text{drug}]/Kd_{closed}}{1+[\text{drug}]/Kd_{open}}\right)^n}$$

where P_{open} is the fraction of receptors that are open in the presence of etomidate or naphthalene–etomidate; [drug] is the concentration of either etomidate or naphthalene–etomidate; Kd_{closed} and Kd_{open}, respectively, are the microscopic dissociation constants of the drug in the closed and open states; and n is the number of drug binding sites. L_0, the closed:open receptor ratio in the absence of drug, was fixed at a median literature value of 40,000.[41,43–46]

Statistical Analysis

At each drug concentration, individual electrophysiologic data points were obtained using different oocytes. Errors bars on mean electrophysiologic data are reported as ± SD, whereas those on mean photoincorporation data are reported as the range of two experiments obtained using two different receptor preparations. Sample sizes (4 to 6 points/drug concentration for electrophysiologic experiments) were defined based on our previous experience.[29,30,47,48] A one-sample t test (two-tailed) was used to statistically assess whether 300 µM naphthalene–etomidate significantly changed peak currents evoked by EC_{50} GABA or EC_5 GABA potentiated by each of the PAMs. The statistical comparisons between the EC_{50} values for propofol potentiation of EC_5 GABA-evoked currents in the presence versus absence of 300 µM naphthalene–etomidate, and those between naphthalene–etomidate potency for inhibiting photolabeling by [³H] azi-etomidate versus R-[³H]mTFD-MPAB were made using the extra sum-of-squares F test. The uncertainties in fitted parameters are reported as CIs. There was no lost or missing data. To avoid output saturation, oocytes producing 1 mM GABA-evoked peak currents greater than 5 µA were discarded. All fitting and statistical tests were performed with GraphPad Prism 6.0h. Statistical significance was assumed for $P < 0.05$.

Results

Potentiation of EC_5 GABA-evoked Currents by Etomidate and Naphthalene–Etomidate

We characterized the effects of etomidate and naphthalene–etomidate over a wide range of concentrations on currents evoked by EC_5 GABA and mediated by $\alpha_1\beta_3\gamma_{2L}$ $GABA_A$ receptors expressed in Xenopus oocytes using the two-electrode voltage clamp technique. Figure 2A (top) shows representative electrophysiologic traces obtained using a single oocyte and demonstrates that etomidate potentiated EC_5 GABA-evoked currents in a concentration-dependent manner. At the highest etomidate concentration shown in that figure (100 µM), the current was potentiated by 14-fold,
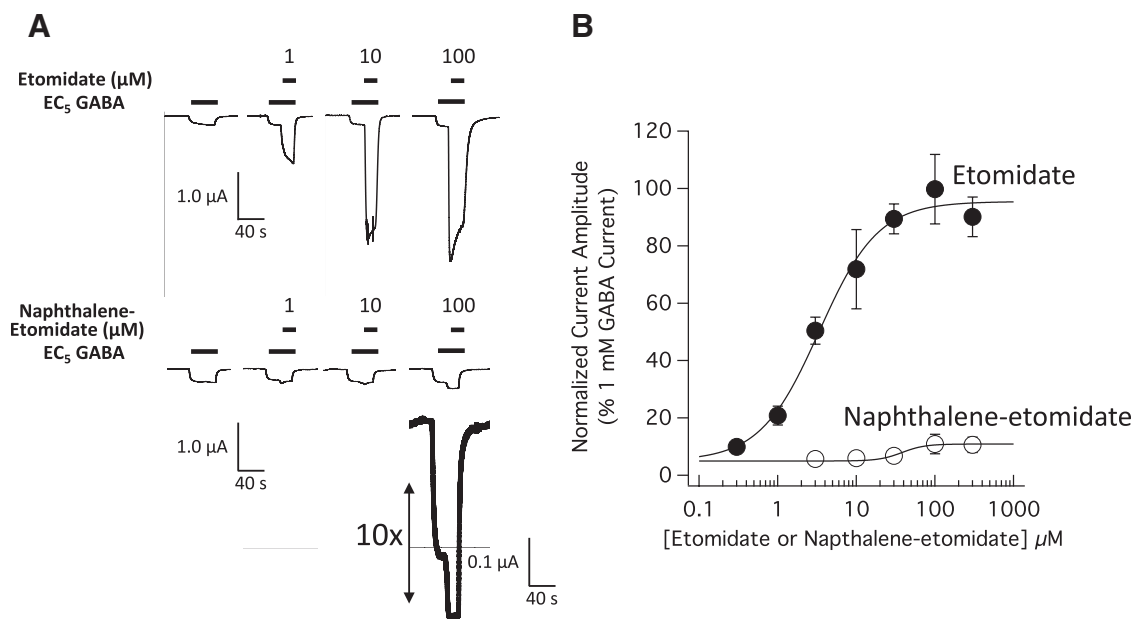

Fig. 2. Potentiation of $\alpha_1\beta_3\gamma_{2L}$ γ-aminobutyric acid type A ($GABA_A$) receptor currents by etomidate and naphthalene–etomidate. (A) Electrophysiologic traces showing the potentiating effect of etomidate (top) or naphthalene–etomidate (bottom) on currents evoked by a γ-aminobutyric acid (GABA) concentration that elicits 5% of the current evoked by 1 mM GABA (EC_5 GABA). For each data set, currents at all drug concentrations were obtained using the same oocyte. (B) Etomidate and naphthalene–etomidate concentration-response curves for potentiation of EC_5 GABA-evoked currents. Each symbol is the mean ± SD derived from six (etomidate) or four (naphthalene–etomidate) different oocytes. The curves are fits of the data sets to equation 1. For etomidate, the fit yielded a half-maximal potentiating concentration of 3.4 µM (95% CI, 2.5 to 4.5 µM), a maximum peak current amplitude at high etomidate concentrations of 95% (95% CI, 89 to 102%) of that produced by 1 mM GABA, and a slope of 1.2 (95% CI, 0.8 to 1.5). For naphthalene–etomidate, the fit yielded a half-maximal potentiating concentration of 38 µM (95% CI, 16 to 93 µM), a maximum peak current value at high concentrations of 11% (95% CI, 8.7 to 13%), and a slope of 3 (95% CI, –5 to 11).

reaching a magnitude that was similar to that evoked by 1 mM GABA (GABA trace not shown). Figure 2A (*bottom*) shows representative electrophysiologic traces obtained using a single oocyte and demonstrates that naphthalene–etomidate also potentiated EC_5 GABA-evoked currents in a concentration-dependent manner. However, the magnitude of potentiation produced by naphthalene–etomidate was relatively small: at most one tenth that produced by the same concentration of etomidate. Figure 2B plots the concentration–mean peak response relationship for potentiation of EC_5 GABA-evoked currents by etomidate (n = 6 oocytes/concentration) and naphthalene–etomidate (n = 4 oocytes/concentration). It shows that etomidate increased peak currents evoked by EC_5 GABA in a manner that was not only potent but also highly efficacious; in the presence of 300 µM etomidate, EC_5-evoked currents that were 90 ± 7% of those evoked by 1 mM GABA. We fit the data to equation 1 with the minimum constrained to 5% (by definition for EC_5 GABA-evoked currents). It yielded an EC_{50} for etomidate potentiation of 3.4 µM (95% CI, 2.5 to 4.5 µM), a maximum peak current amplitude at high etomidate concentrations that was 95% (95% CI, 89 to 102%) of that produced by 1 mM GABA, and a slope of 1.2 (95% CI, 0.8 to 1.5). Figure 2B also shows that naphthalene–etomidate was significantly less potent and efficacious than etomidate; in the presence of 300 µM naphthalene–etomidate (a near aqueous-saturating concentration), EC_5-evoked currents were only 11 ± 2.7% of those evoked by 1 mM GABA. A fit of the naphthalene–etomidate data to equation 1 yielded an EC_{50} value for naphthalene–etomidate potentiation of 38 µM (95% CI, 16 to 93 µM), a maximum peak current value at high concentrations of only 11% (95% CI, 8.7 to 13%), and a slope of 3 (95% CI, –5 to 11).

Direct Activation of GABA_A Receptor Currents by Etomidate and Naphthalene–Etomidate

We then compared the abilities of etomidate and naphthalene–etomidate to directly activate $\alpha_1\beta_3\gamma_{2L}$ GABA_A receptor currents in the absence of GABA. Using the same oocyte for etomidate and naphthalene–etomidate, figure 3A shows that currents directly activated by naphthalene–etomidate (*bottom*) are orders of magnitude smaller than those activated by etomidate (*top*). Figure 3B plots the concentration-mean peak response relationship for direct activation by etomidate (n = 4 oocytes/concentration) and naphthalene–etomidate (n = 4 oocytes/concentration). A fit of the etomidate data to equation 1 with the minimum constrained to 0% (*i.e.*, no current in the absence of drug) yielded an EC_{50} for direct activation, maximum current amplitude at high concentrations, and slope of 65 µM (95% CI, 41 to 103 µM) and 54% (95% CI, 46 to 63%), and 1.2 (95% CI, 0.7 to 1.8), respectively. An analogous fit of the naphthalene–etomidate data set failed to converge.

Naphthalene–Etomidate Inhibits GABA_A Receptor Photolabeling by [³H]Azi-etomidate and R-[³H]mTFD-MPAB

To test whether naphthalene–etomidate bound to either (or both) classes of transmembrane anesthetic binding sites, we quantified its ability to inhibit photoaffinity labeling of $\alpha_1\beta_3\gamma_{2L}$ GABA_A receptors by [³H]azi-etomidate and the barbiturate R-[³H]mTFD-MPAB. Figure 4 shows that naphthalene–etomidate reduced photoincorporation of both [³H]azi-etomidate and R-[³H]mTFD-MPAB in a concentration-dependent manner. At the highest concentrations studied, naphthalene–etomidate inhibited specific (*i.e.*, etomidate-displaceable) [³H]azi-etomidate photoincorporation and specific (*i.e.*, R-mTFD-MPAB-displaceable) R-[³H]mTFD-MPAB photoincorporation by at
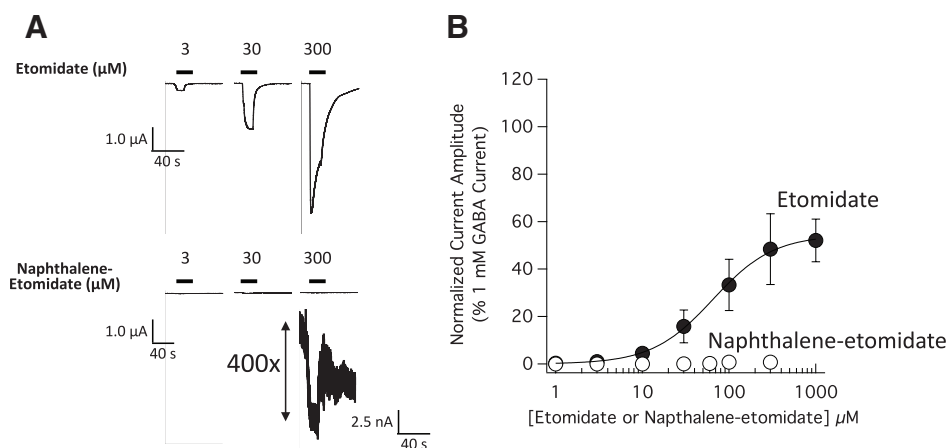
Fig. 3. Direct activation of $\alpha_1\beta_3\gamma_{2L}$ γ-aminobutyric acid type A (GABA_A) receptor currents by etomidate and naphthalene–etomidate. (*A*) Electrophysiologic traces showing the direct activation by etomidate (*top*) or naphthalene–etomidate (*bottom*). To allow a direct comparison between drugs, a single oocyte was used to obtain both data sets. (*B*) Etomidate and naphthalene–etomidate concentration-response curves for direct activation. Each symbol is the mean ± SD derived from four different oocytes. The curve is a fit of the etomidate data set to equation 1 yielding a half-maximal direct activating concentration, maximum current amplitude at high etomidate concentrations, and slope of 65 µM (95% CI, 41 to 103 µM) and 54% (95% CI, 46 to 63%), and 1.2 (95% CI, 0.7 to 1.8), respectively. A fit of the naphthalene–etomidate data set to equation 1 did not converge.
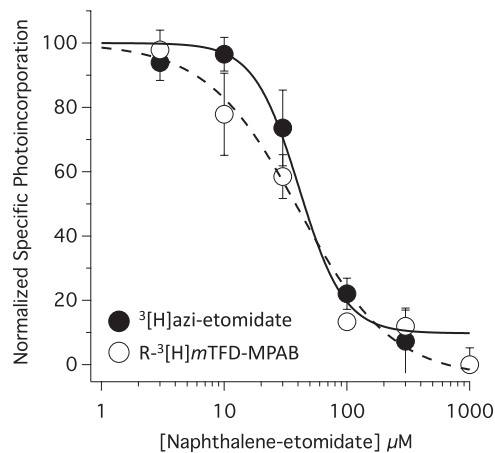
Fig. 4. Naphthalene–etomidate concentration-response curves for inhibition of specific [³H]azi-etomidate and R-[³H]*m*TFD-MPAB photolabeling of $\alpha_1\beta_3\gamma_{2L}$ γ-aminobutyric acid type A (GABA$_A$) receptors. For the two photolabels, the half-maximal inhibitory concentrations of naphthalene–etomidate were 48 μM (95% CI, 28 to 81 μM) and 33 μM (95% CI, 20 to 54 μM), respectively. The slopes were –2.0 (95% CI, –3.5 to –0.5) and –1.3 (95% CI, –2.0 to –0.6), respectively. The data were normalized to counts/min measured in the absence of naphthalene–etomidate. Nonspecific photolabeling was defined in the presence of 300 μM etomidate (for [³H]azi-etomidate photolabeling experiments) or 100 μM R-*m*TFD-MPAB (for R-[³H]*m*TFD-MPAB photolabeling experiments). All photolabeling was done in the presence of 300 μM γ-aminobutyric acid (GABA).

least 90%. Naphthalene–etomidate IC$_{50}$ values for inhibiting specific photoincorporation of the two photolabels were 48 μM (95% CI, 28 to 81 μM) for [³H]azi-etomidate and 33 μM (95% CI, 20 to 54 μM) for R-[³H]*m*TFD-MPAB with respective slopes of –1.2 (95% CI, –2.0 to –0.6) and –2.0 (95% CI, –3.5 to –0.5). These IC$_{50}$ values were not significantly different from one another, strongly suggesting that naphthalene–etomidate binds to both classes of transmembrane anesthetic binding sites on the GABA$_A$ receptor with similar affinities.

Naphthalene–Etomidate Antagonizes Anesthetic Potentiated GABA$_A$ Receptor Currents

The observation that naphthalene–etomidate inhibits photoaffinity labeling of the transmembrane anesthetic binding sites of the GABA$_A$ receptor (most likely because it binds to these sites) but has very low efficacy for positively modulating GABA$_A$ receptor function suggested to us that it might be capable of acting as a competitive antagonist of anesthetics that also bind to these sites. An analogous competitive mechanism at the classical benzodiazepine binding site of the GABA$_A$ receptor (located at the extracellular α^+–γ^- subunit interface) accounts for the ability of flumazenil to reverse the GABA$_A$ receptor actions of benzodiazepines.[35] To test this possibility, we assessed whether naphthalene–etomidate would reduce the agonist potentiating effects of etomidate, propofol, and pentobarbital. As control experiments, we also assessed the actions of naphthalene–etomidate on receptors similarly potentiated by diazepam or activated with an EC$_{50}$ GABA concentration.

In one set of studies, we simultaneously applied naphthalene–etomidate (300 μM) along with either (1) EC$_{50}$ GABA alone or (2) EC$_5$ GABA plus a PAM (etomidate, propofol, pentobarbital, or diazepam). Concentrations of etomidate (3 μM), propofol (10 μM), and pentobarbital (120 μM) were chosen based on pilot experiments indicating that when combined with EC$_5$ GABA, they activate the same fraction of GABA$_A$ receptors as EC$_{50}$ GABA alone. We used a diazepam concentration of 1 μM to maximally—but selectively—potentiate GABA$_A$ receptors *via* the classical extracellular benzodiazepine binding site.[49] Representative current traces recorded during these experiments are shown in figure 5. Figure 5A shows electrophysiologic traces obtained upon perfusing an oocyte with either EC$_{50}$ GABA alone or EC$_{50}$ GABA plus 300 μM naphthalene–etomidate. It demonstrates that 300 μM naphthalene–etomidate minimally affected currents evoked by EC$_{50}$ GABA. In contrast, figures 5B through 5E) respectively show that 300 μM naphthalene–etomidate reduced EC$_5$ GABA$_A$ receptor currents that were potentiated by etomidate, propofol, or pentobarbital but enhanced those potentiated by diazepam. The change in peak currents produced by naphthalene–etomidate upon activation with either EC$_{50}$ GABA alone or EC$_5$ GABA along with each of the four PAMs (n = 6 oocyte experiments/drug) is plotted in figure 5F with the mean values summarized in table 1 in the simultaneous addition row.

In a second set of studies, we preapplied 300 μM naphthalene–etomidate for 10 s before activating with either EC$_{50}$ GABA alone or EC$_5$ GABA plus a PAM (fig. 6). The results were similar to those described in the previous paragraph using the simultaneous addition protocol with naphthalene–etomidate having no significant effect on peak currents activated by EC$_{50}$ GABA while significantly reducing EC$_5$ GABA-evoked currents potentiated by etomidate, propofol, or pentobarbital and enhancing EC$_5$ GABA-evoked currents potentiated by diazepam. The mean values for these studies are summarized in table 1 in the naphthalene–etomidate preexposure row.

In a third set of studies, we first activated GABA$_A$ receptors using either EC$_{50}$ GABA alone or EC$_5$ GABA plus a PAM before adding 300 μM naphthalene–etomidate (fig. 7). With this protocol, we observed significant potentiation during naphthalene–etomidate administration when receptors were activated by either EC$_{50}$ GABA alone or EC$_5$ GABA plus diazepam. However, naphthalene–etomidate again significantly reduced EC$_5$ GABA-evoked currents potentiated by etomidate, propofol, or pentobarbital. The mean values for these studies are summarized in table 1 in the GABA preexposure row.

Naphthalene–Etomidate Rightward Shifts the Propofol Concentration-response Curve for EC$_5$ GABA Potentiation

We then assessed the impact of naphthalene–etomidate on the anesthetic concentration-response curve for EC$_5$ potentiation using the simultaneous addition protocol. We chose to study propofol as a representative anesthetic
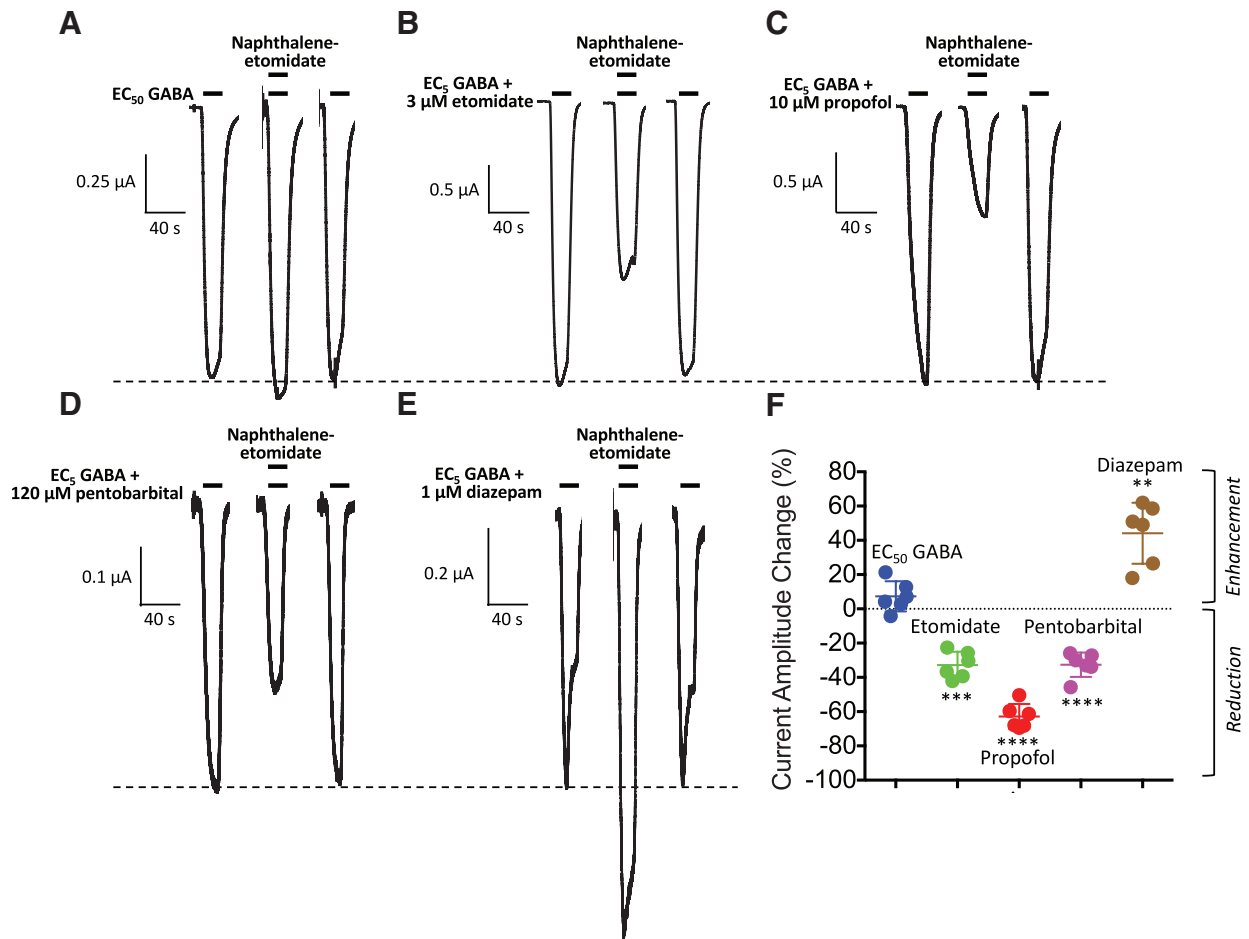
Fig. 5. Naphthalene–etomidate modulation of $\alpha_1\beta_3\gamma_{2L}$ γ-aminobutyric acid type A (GABA$_A$) receptor currents: simultaneous addition protocol. (*A*) Representative current traces obtained upon application of γ-aminobutyric acid (GABA) at a concentration that evokes 50% of the current evoked by 1 mM GABA (EC$_{50}$ GABA). The first and last traces were controls obtained in the absence of naphthalene–etomidate, and the middle trace was obtained with simultaneous addition of 300 μM naphthalene–etomidate along with GABA. (*B–E*) Representative current traces obtained upon application of GABA at a concentration that evokes 5% of the current evoked by 1 mM GABA (EC$_5$ GABA) along with the indicated positive allosteric modulator. In each panel, the first and last traces were controls obtained in the absence of naphthalene–etomidate, and the middle trace was obtained with simultaneous addition of 300 μM naphthalene–etomidate along with GABA + modulator. In each panel, the *dashed line* shows the average control peak current produced in the absence of naphthalene–etomidate. (*F*) Percent change in peak current amplitude produced by 300 μM naphthalene–etomidate. Positive values indicate that naphthalene–etomidate enhanced peak currents, whereas negative values indicate that it reduced peak currents. Each symbol represents data from a single oocyte experiment (n = 6 oocyte experiments/drug). Means ± SD are indicated for each data set. Statistically significant change in current amplitude was produced by naphthalene–etomidate. **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

not only because it is the most widely used anesthetic, but also because our data showed that naphthalene–etomidate produced an approximately twofold greater reduction in currents potentiated by propofol as compared to currents potentiated by etomidate or pentobarbital (table 1). Thus, we expected that any change in the anesthetic concentration-response curve produced by naphthalene–etomidate would be greater if we used propofol to potentiate currents rather than etomidate or pentobarbital. Figure 8A shows the propofol concentration-response relationship for EC$_5$ potentiation in the absence and presence of 300 μM naphthalene–etomidate. The curves are fits of the two data sets to equation 1. In the absence and presence of

naphthalene–etomidate, the respective minima were constrained to 5% (by definition for EC$_5$ GABA evoked currents) and 11% (the peak current amplitude produced by 300 μM naphthalene–etomidate in the absence of propofol). The EC$_{50}$ for propofol potentiation of EC$_5$ GABA-evoked currents was 6.0 μM (95% CI, 4.4 to 8.0 μM) in the absence of naphthalene–etomidate and increased sixfold to 36 μM (95% CI, 17 to 78 μM) in its presence. The maximal peak current response at high propofol concentrations was virtually unchanged by 300 μM naphthalene–etomidate with values of 88% (95% CI, 81 to 96%) in the absence of naphthalene–etomidate and 87% (95% CI, 66 to 107%) in its presence.

Table 1. Percent Change in γ-Aminobutyric Acid Type A (GABA$_A$) Receptor Peak Current Amplitude Produced by 300 μM Naphthalene–Etomidate

Protocol	GABA EC$_{50}$	GABA EC$_5$ + Etomidate	GABA EC$_5$ + Propofol	GABA EC$_5$ + Pentobarbital	GABA EC$_5$ + Diazepam
Simultaneous addition	7.3 ± 8.8	-33 ± 7.8	-63 ± 7.3	-33 ± 7.1	44 ± 18
Naphthalene–etomidate preexposure	-8.8 ± 8.6	-47 ± 4.1	-68 ± 6.0	-45 ± 7.9	25 ± 14
GABA preexposure	21 ± 9	-24 ± 1.8	-49 ± 4.69	-21 ± 5.4	33 ± 6.9

All values are means ± SD (n = 6 oocytes). GABA = γ-aminobutyric acid; EC$_5$ = the GABA concentration that elicits 5% of the current evoked by 1 μM GABA; EC$_{50}$ = the GABA concentration that elicits 50% of the current evoked by 1 μM GABA.
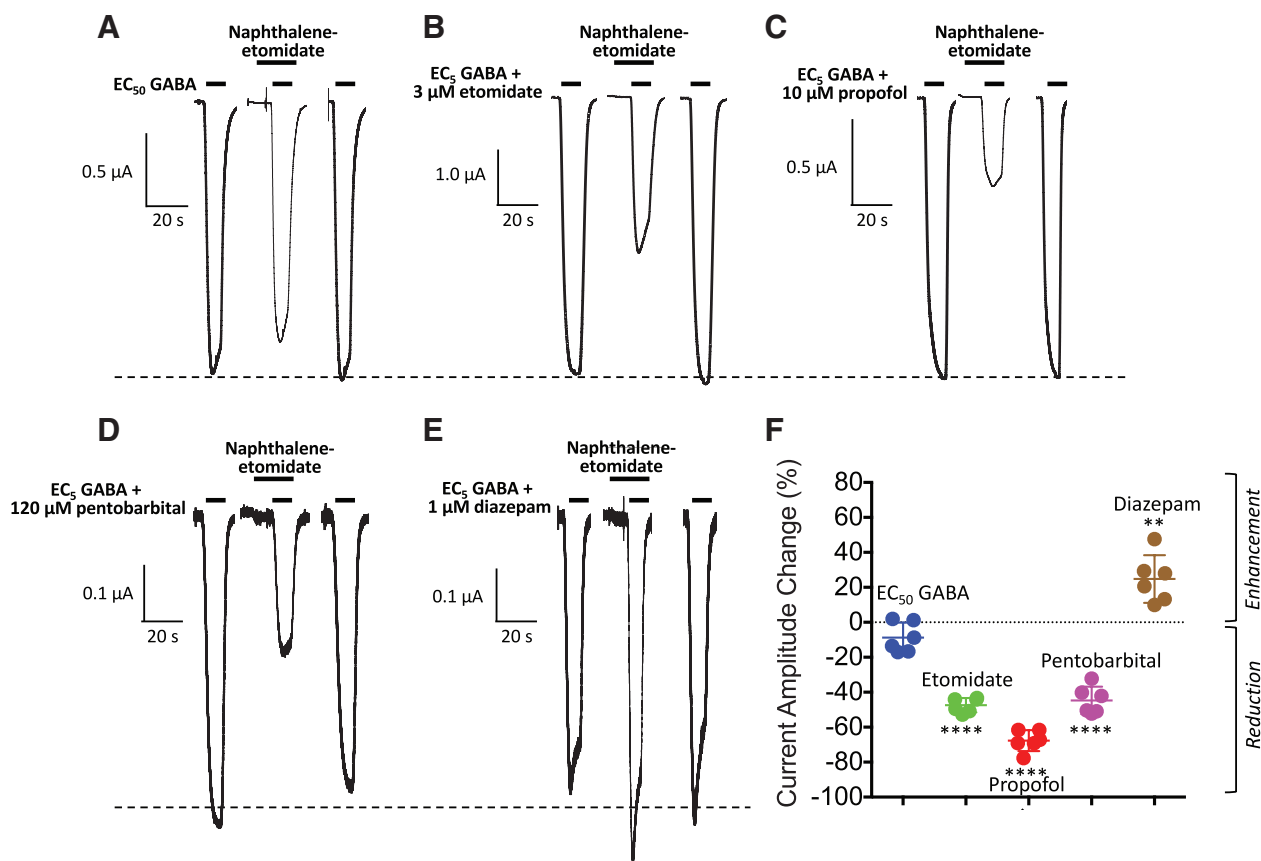
Fig. 6. Naphthalene–etomidate modulation of $\alpha_1\beta_3\gamma_{2L}$ γ-aminobutyric acid type A (GABA$_A$) receptor currents: naphthalene–etomidate preexposure protocol. (*A*) Representative current traces obtained upon application of γ-aminobutyric acid (GABA) at a concentration that evokes 50% of the current evoked by 1 mM GABA (EC$_{50}$ GABA). The first and last traces were controls obtained in the absence of naphthalene–etomidate, and the middle trace was obtained with a 10-s preexposure of 300 μM naphthalene–etomidate along with GABA. (*B–E*) Representative current traces obtained upon application of GABA at a concentration that evokes 5% of the current evoked by 1 mM GABA (EC$_5$ GABA) along with the indicated positive allosteric modulator. In each panel, the first and last traces were controls obtained in the absence of naphthalene–etomidate, and the middle trace was obtained with a 10-s preexposure of 300 μM naphthalene–etomidate. In each panel, the *dashed line* shows the average control peak current produced in the absence of naphthalene–etomidate. (*F*) Percent change in peak current amplitude produced by 300 μM naphthalene–etomidate. Positive values indicate that naphthalene–etomidate enhanced peak currents, whereas negative values indicate that it reduced peak currents. Each symbol represents data from a single oocyte experiment (n = 6 oocyte experiments/drug). Means ± SD are indicated for each data set. Statistically significant change in current amplitude was produced by naphthalene–etomidate: **$P < 0.01$; ****$P < 0.0001$.

Naphthalene–Etomidate Antagonizes EC$_5$ GABA Potentiation by Propofol in a Concentration-dependent Manner

Again using the simultaneous addition protocol, we defined the naphthalene–etomidate concentration dependence for inhibiting the peak amplitude of EC$_5$ GABA-evoked currents potentiated by 10 μM propofol. We found that the peak amplitude of propofol-potentiated currents decreased steeply with naphthalene–etomidate concentration (fig. 8B).
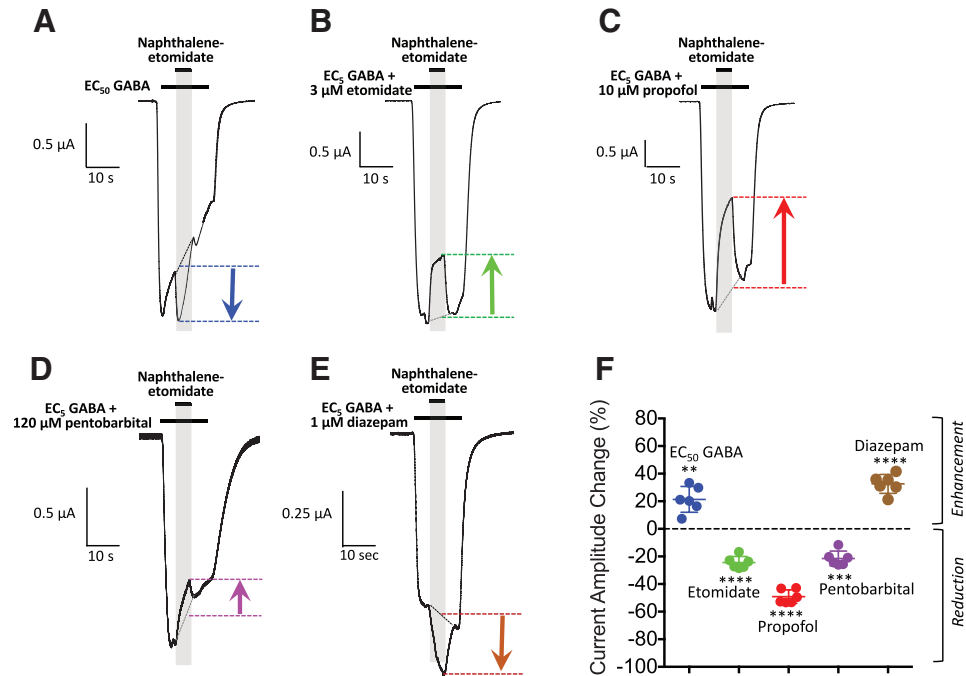
Fig. 7. Naphthalene–etomidate modulation of $\alpha_1\beta_3\gamma_{2L}$ γ-aminobutyric acid type A (GABA$_A$) receptor currents: γ-aminobutyric acid (GABA) preexposure protocol. (*A*) Representative current trace obtained upon receptor activation for 30 s with GABA at a concentration that evokes 50% of the current evoked by 1 mM GABA (EC$_{50}$ GABA). Ten seconds into this activation period, 300 μM naphthalene–etomidate was added for 10 s. (*B–E*) Representative current trace obtained upon receptor activation for 30 s with GABA at a concentration that evokes 5% of the current evoked by 1 mM GABA (EC$_5$ GABA) along with the indicated positive allosteric modulator. Ten seconds into this activation period, 300 μM naphthalene–etomidate was added for 10 s. (*F*) Percent change in peak current amplitude produced by 300 μM naphthalene–etomidate. Positive values indicate that naphthalene–etomidate enhanced peak currents, whereas negative values indicate that it reduced peak currents. Each symbol represents data from a single oocyte experiment (n = 6 oocyte experiments/drug). Means ± SD are indicated for each data set. Statistically significant change in current amplitude was produced by naphthalene–etomidate: **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.
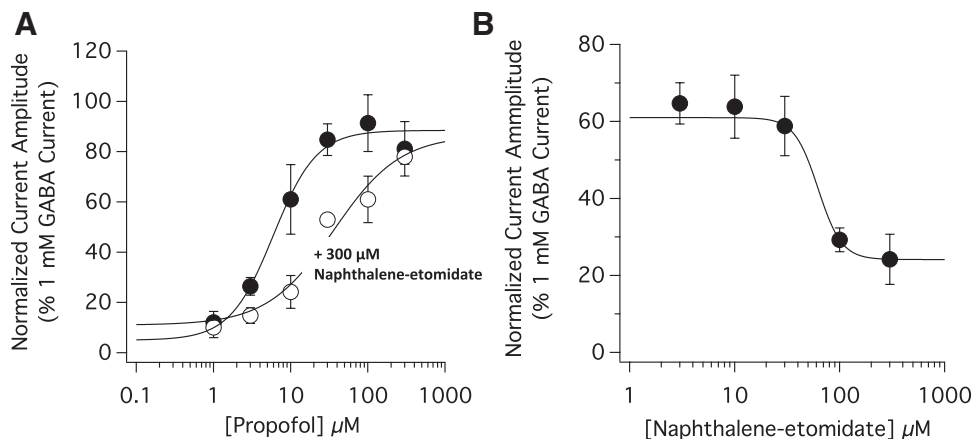

Fig. 8. Inhibition of propofol-mediated potentiation of $\alpha_1\beta_3\gamma_{2L}$ γ-aminobutyric acid type A (GABA$_A$) receptor currents by naphthalene–etomidate. (*A*) Propofol concentration-response curves for potentiation of γ-aminobutyric acid (GABA)-evoked currents in the absence and presence of 300 μM naphthalene–etomidate. The curves are fits of the data sets to equation 1. The propofol concentration that half-maximally potentiated GABA-evoked currents (EC$_{50}$) was 6.0 μM (95% CI, 4.4 to 8.0 μM) in the absence of naphthalene–etomidate and 36 μM (95% CI, 17 to 78 μM) in the presence of 300 μM etomidate. The respective slopes were 1.5 (95% CI, 0.97 to 2.1) and 1.0 (95% CI, 0.54 to 1.5 μM). In the absence and presence of 300 μM naphthalene–etomidate, the maximal responses at high propofol concentrations were essentially identical with values of 88% (95% CI, 81 to 96%) and 87% (95% CI, 66 to 107%), respectively. (*B*) Naphthalene–etomidate concentration-response curves for inhibition of GABA-evoked currents potentiated by 10 μM propofol. The curves are fits of the data sets to equation 2. The naphthalene–etomidate concentration that half-maximally inhibited potentiated currents (IC$_{50}$) was 62 μM (95% CI, 38 to 103 μM) with a minimum value of 24% (95% CI, 17 to 31%) and a slope of –3.9 (95% CI, –9.7 to –0.4). In both panels, each data point is the mean ± SD of four oocyte experiments.

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A fit of this relationship to equation 2 with the maximum constrained to 61% (the peak current amplitude produced by 10 μM propofol in the absence of naphthalene–etomidate) yielded an IC_{50} of 62 μM (95% CI, 38 to 103 μM), a minimum value of 24% (95% CI, 17 to 31%), and a slope of −3.9 (95% CI, −9.7 to −0.4).

Discussion

This report describes a novel etomidate analog that exhibits the pharmacology of an anesthetic-selective competitive antagonist. Specifically, our studies show that naphthalene–etomidate (1) inhibits photoaffinity labeling of the two classes of $GABA_A$ receptor transmembrane anesthetic binding sites with similar affinities but possesses low intrinsic efficacy for positively modulating $GABA_A$ receptor function, (2) reduces the positive modulatory actions of drugs that bind to these receptor sites (propofol > etomidate ~ pentobarbital) but not those of drugs that bind elsewhere on the receptor (GABA and diazepam), and (3) shifts the anesthetic (propofol) concentration-response curve for potentiation rightward without affecting the maximal response obtained at high anesthetic concentrations.

Within the context of Monod–Wyman–Changeux allosteric models of receptor function, the intrinsic efficacy of a ligand is defined by its relative affinity for the open *versus* closed receptor states (fig. 9A).[43,50,51] Ligands with high efficacies bind with much higher affinity to the open state than to the closed state. Such state-selective binding shifts the preexisting receptor equilibrium between closed and open states toward the open state. For propofol and etomidate, the relative binding affinities for the open *versus* closed states of the $GABA_A$ receptor have been estimated to be on the order of 100:1, quantitatively accounting for both their agonist potentiating and direct activating actions.[41,44]

In contrast, ligands with very low intrinsic efficacies (*i.e.*, competitive antagonists) bind with similar affinities to both receptor states.[52] Consequently, they minimally perturb the closed:open state equilibrium and have little functional effect on their own. However, they can competitively inhibit the binding—and thus the actions—of more efficacious ligands.

The affinities of a ligand for the open and closed states can be quantified from the relationship between the ligand concentration and the fraction of receptors that it opens (P_{open}). For the allosteric model shown in figure 9A, this relationship is defined by equation 3. Figure 9B plots that relationship using the direct activation data for etomidate and naphthalene–etomidate where P_{open} was determined at each drug concentration from the peak amplitude of the directly activated current normalized to that evoked by a maximally activating GABA concentration (*i.e.*, 1 mM) and assuming a maximum P_{open} value for GABA of 0.85 in this receptor subtype.[42] The curved lines in this figure are fits of this relationship to equation 3 with the number of anesthetic binding sites n constrained to 2 for etomidate (at the two β^+–α^- subunit interfacial sites) and 4 for naphthalene–etomidate (because our photoaffinity labeling studies suggest that it binds to the two β^+– α^- subunit interfacial sites and to the α^+–β^- and γ^+–β^- interfacial sites with similar affinities).[31,45,51] Based on this analysis, we determined that etomidate binds to the open state with an affinity that is 190-fold higher than to the closed state with microscopic dissociation constants of 0.23 μM (95% CI, 0.15 to 0.31 μM) and 44 μM (95% CI, 26 to 62 μM), respectively. In contrast, naphthalene–etomidate binds to the open state with an affinity that is only 4.4-fold higher than to the closed state with microscopic dissociation constants of 6.2 and 27 μM, respectively. Comparison of the dissociation constants for the two drugs reveals that the much lower intrinsic efficacy
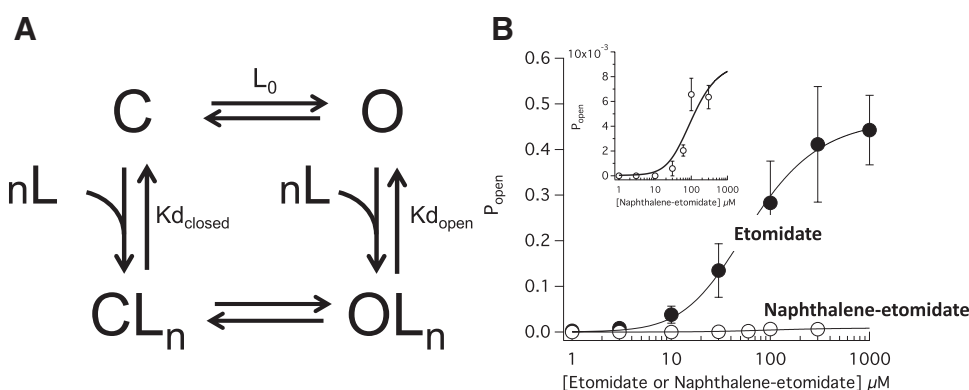
Fig. 9. Allosteric analysis of $\alpha_1\beta_3\gamma_{2L}$ γ-aminobutyric acid type A ($GABA_A$) receptor direct activation by etomidate and naphthalene–etomidate. (*A*) Allosteric model for receptor activation. *C* and *O* are the closed and open states, respectively. CL_n and OL_n are the liganded closed and open states, respectively, and *n* is the number of ligand (etomidate or naphthalene–etomidate) binding sites. L_0 is the open state:closed state ratio in the absence of any modulatory ligands. Kd_{closed} and Kd_{open} are the ligand microscopic dissociation constants in the closed and open states, respectively. (*B*) $GABA_A$ receptor open state probability (P_{open}) as a function of etomidate or naphthalene–etomidate concentration. The *inset* shows the naphthalene–etomidate data on an expanded vertical axis. The curves are fits of the data sets to equation 3 yielding respective Kd_{closed} and Kd_{open} values of 0.23 μM (95% CI, 0.15 to 0.31 μM) and 44 μM (95% CI, 26 to 62 μM) for etomidate and 6.2 μM and 27 μM for naphthalene–etomidate. For etomidate and naphthalene–etomidate, the number of binding sites (n) was assumed to be 2 and 4, respectively. L_0 was constrained at 40,000 for both fits.

of naphthalene–etomidate can be almost entirely attributed to a 27-fold lower affinity of for the open state of the receptor.

Our studies also showed that naphthalene–etomidate inhibited photoaffinity labeling by [³H]azi-etomidate and R-[³H]mTFD-MPAB with similar IC$_{50}$ values. This suggests that in addition to markedly reducing binding selectivity for the open *versus* closed receptor, adding the phenyl substituent group also abolished the 100-fold binding selectivity that etomidate has to the receptor's two β$^+$–α$^-$ sites *versus* its α$^+$–β$^-$/γ$^+$–β$^-$ sites. Thus, the selectivity of naphthalene–etomidate for these two classes of transmembrane anesthetic binding sites more closely resembles that of propofol (which has virtually no binding site selectivity) and other phenyl-substituted etomidate analogs than etomidate or pentobarbital (which respectively bind selectively to the receptor's two β$^+$–α$^-$ sites and to its α$^+$–β$^-$/γ$^+$–β$^-$ sites).[32]

To examine the impact of naphthalene–etomidate on currents activated by EC$_{50}$ GABA alone or by EC$_5$ GABA potentiated by various PAMs, we utilized three protocols that added naphthalene–etomidate at different times relative to receptor activation (before, after, or during activation). In general, the effect was similar regardless of when naphthalene–etomidate was added (figs. 5F, 6F, and 7F, and table 1). In all three cases, naphthalene–etomidate significantly reduced EC$_5$ GABA-evoked currents potentiated by etomidate, propofol, or pentobarbital but enhanced those potentiated by diazepam. Additionally, the inhibitory effects of naphthalene–etomidate on propofol-potentiated currents were approximately twice as large as those on etomidate-potentiated and pentobarbital-potentiated currents. The effects of naphthalene–etomidate on EC$_{50}$ GABA-evoked currents were somewhat more protocol-dependent as it had little or no effect when added before or simultaneously with GABA application but significantly potentiated currents when added after GABA application.

The hallmark of a competitive antagonist is that it produces a rightward shift in the concentration-response curve of the drug with which it competes (*i.e.*, it increases the drug's EC$_{50}$) without reducing the maximal response produced by the drug at high concentrations. This may be contrasted with the effects of noncompetitive antagonists that classically reduce the maximal response without shifting the concentration-response curve. Our data show that naphthalene–etomidate exhibits the pharmacology of an anesthetic competitive antagonist as it increased the EC$_{50}$ for propofol potentiation of EC$_5$ GABA-evoked currents by sixfold without reducing the maximal response recorded at high propofol concentrations. Although not tested, we expect that naphthalene–etomidate would have similarly shifted the concentration-response curves for etomidate and pentobarbital as they also bind to the transmembrane anesthetic binding sites on the GABA$_A$ receptor. However, the magnitude of those shifts would almost certainly have been smaller because the inhibitory actions of naphthalene–etomidate on etomidate-potentiated and pentobarbital-potentiated currents are less than those on propofol-potentiated ones.

Based on our experimental observations, we propose the following conceptual model to explain key results of our studies (fig. 10). In the absence of any other positive modulatory ligands, naphthalene–etomidate binds to the two classes of transmembrane anesthetic binding sites and very modestly (because it has low intrinsic efficacy) positively modulates receptors (fig. 10A). In the presence of positive modulatory ligands that act at sites other than these anesthetic binding sites (*e.g.*, GABA and diazepam), naphthalene–etomidate similarly binds and modestly positively modulates receptors (fig. 10, B and C). However, when a general anesthetic is bound to a transmembrane anesthetic binding site and positively modulating the GABA$_A$ receptor, the *net* effect of naphthalene–etomidate binding to that site is inhibitory because it displaces the higher efficacy anesthetic. In the case of propofol (which positively modulates by binding to both classes of anesthetic binding sites), naphthalene–etomidate has the greatest inhibitory effect on potentiated currents because it displaces anesthetic binding from both classes of anesthetic binding sites (fig. 10D). Thus, the net effect of naphthalene–etomidate binding to each of the two classes of anesthetic binding sites is inhibitory. In the case of etomidate (which positively modulates by binding to the β$^+$–α$^-$ anesthetic binding sites), naphthalene–etomidate has two opposing effects on receptor function (fig. 10E). It has an inhibitory effect by displacing etomidate from the β$^+$–α$^-$ anesthetic binding sites. However, it also has a modest positive modulatory effect by binding to the unoccupied α$^+$–β$^-$/γ$^+$–β$^-$ anesthetic binding sites. The result of these opposing actions at the two classes of sites is that naphthalene–etomidate is only about half as effective at reducing etomidate-potentiated currents as propofol-potentiated currents. The case of pentobarbital (which positively modulates by binding to the α$^+$–β$^-$/γ$^+$–β$^-$ anesthetic binding sites) is identical to that of etomidate except that actions at the two classes of anesthetic binding sites are exactly reversed (fig. 10F). Although our model does not explicitly include the possibility of other anesthetic binding sites besides those identified by [³H]azi-etomidate and R-[³H]mTFD-MPAB photolabeling, it does not provide evidence against their existence as naphthalene–etomidate may bind to these sites as well.

There are several potential clinical and experimental uses for anesthetic competitive antagonists. Currently, recovery from anesthesia must occur as a passive process whose time course is dictated by the rate of anesthetic drug clearance rather than the actual clinical need. The development of competitive antagonists for general anesthetics that act *via* the GABA$_A$ receptor could change this paradigm if they allow anesthesia to be reversed immediately and on demand. This direct competitive approach may be contrasted with ones that utilize stimulants that target other proteins and achieve emergence from anesthesia presumably by producing central nervous system arousal/stimulation.[5,53–55] Beyond their potential clinical utility as anesthetic reversal agents, members of this new class of drugs would also be extremely valuable research tools. They would help scientists locate functionally
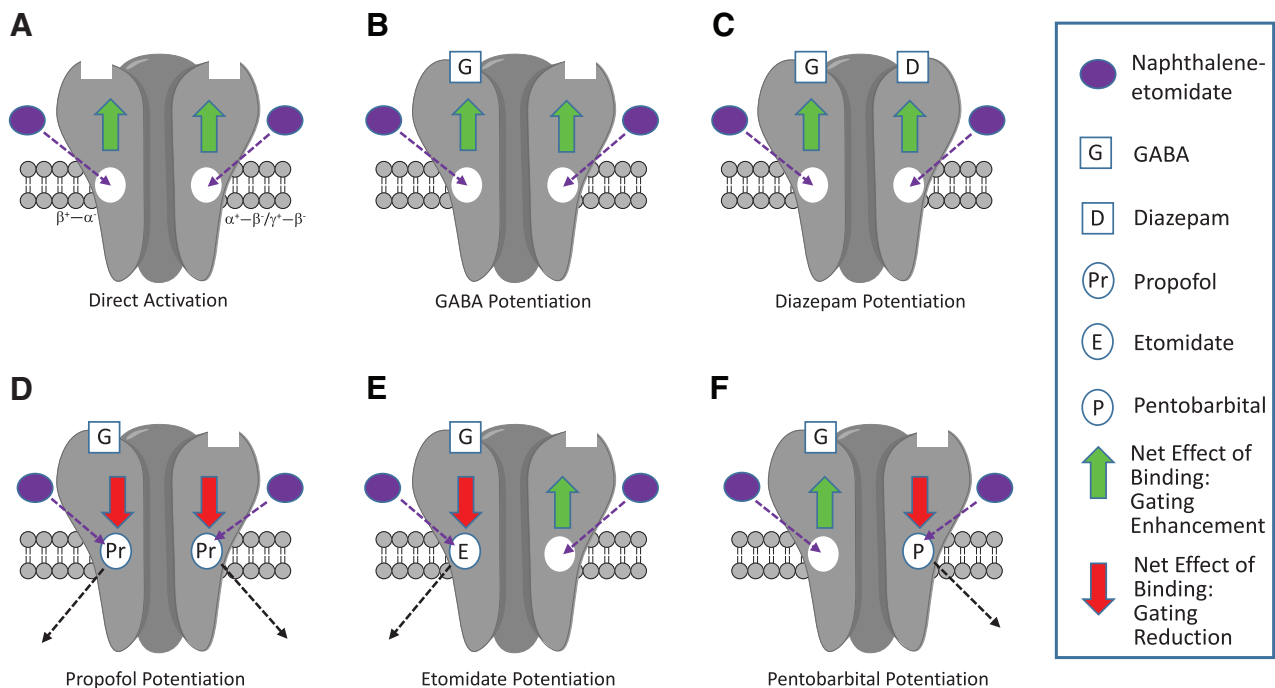
Fig. 10. Conceptual model of the actions of naphthalene–etomidate on γ-aminobutyric acid type A (GABA_A) receptor pharmacology. The key features are that naphthalene–etomidate (1) binds to both classes of transmembrane anesthetic binding sites; (2) has lower intrinsic positive modulatory efficacy than propofol, etomidate, and pentobarbital; and (3) competitively antagonizes the binding of these three anesthetics, but not γ-aminobutyric acid (GABA) or diazepam, because they bind elsewhere. Thus, when the transmembrane anesthetic binding sites are unoccupied (*A–C*), naphthalene–etomidate weakly enhances channel gating (*green arrows*). However, when such sites are occupied by an anesthetic possessing higher efficacy (*D–F*), the net effect of naphthalene–etomidate binding to that site (and competitively displacing the anesthetic) is to reduce gating efficacy. The inhibitory effect of naphthalene–etomidate on currents potentiated by propofol (*D*) is greater than currents potentiated by either etomidate (*E*) or pentobarbital (*F*) because the latter two anesthetics bind selectively to only one class of sites. This allows naphthalene–etomidate to bind to the other (unoccupied) class of sites where its effect is to enhance channel gating efficacy.

important anesthetic binding sites on GABA_A receptors, rationally design new exogenous ligands for these sites, and define the role that GABA_A receptors play in producing particular anesthetic pharmacologic, physiologic, toxicologic, and behavioral actions. Naphthalene–etomidate provides proof of concept for the design of anesthetic analogs with low intrinsic efficacies that may act as anesthetic-selective competitive antagonists at receptor targets. Such compounds would be valuable drugs for patient care and pharmacologic tools to define the mechanisms of anesthetic action.

Competing Interests

The authors declare no competing interests.

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ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

Chicago Art Institute Alumnus Dalton Stevens Covers a Criminal Chloroforming

The administration of ether and of chloroform were associated with anesthetic morbidity and mortality severe enough to inspire the search for alternative inhalational anesthetics. Ethylene and then cyclopropane were added to the anesthetic armamentarium in the mid-1920s and the mid-1930s, respectively. However, another driving force behind abandoning chloroform was the anesthetic's criminal use in assaults, kidnappings, and murders, many of which were sensationalized in news reports and pulp fiction during the first half of the twentieth century. One classically trained artist who turned to illustrating covers of popular "pulp" magazines was Virginia native E. Dalton Stevens (1878 to 1939). After attending the Art Institute of Chicago, Stevens moved to New York and eventually to New Jersey. He illustrated covers, particularly for adventure and detective magazines. Plagued by gradually worsening hearing and vision, Stevens managed to compose the cover image (*above*) of a criminal chloroforming. Despondent over his blindness, the 61-yr-old artist finally committed suicide in 1939, not by (chloroform) rag but by bullet. (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

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