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The Effects of a Competitive Antagonist on GABA-evoked Currents in the Presence of Sedative-Hypnotic Agents

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Abstract

Background—Many sedative-hypnotic agents are thought to act by positively modulating γ -aminobutyric acid type A (GABA_A) receptors. However, for many agents, the location(s) of the binding site(s) responsible for such receptor modulation is uncertain. We previously developed a low efficacy ligand (naphthalene-etomidate) that binds within a homologous set of hydrophobic cavities located at GABA_A receptor subunit interfaces in the transmembrane domain and thus acts as a competitive antagonist for higher efficacy sedative-hypnotics that also bind to these sites. In this report, we describe studies using this compound as a pharmacological screening tool to test whether sedative-hypnotics representing a range of chemical classes can modulate GABA_A receptors by binding within these receptor cavities.

Methods—The impact of naphthalene-etomidate on GABA-evoked currents that were mediated by oocyte-expressed $\alpha_1\beta_3\gamma_{2L}$ GABA_A receptors and potentiated by muscimol, alphaxalone, 2,2,2-trichloroethanol, isoflurane, AA29504, loreclezole, or diazepam was quantified using electrophysiological techniques.

Results—Naphthalene-etomidate (300 μ M) significantly reduced GABA_A receptor currents potentiated by alphaxalone (by $22 \pm 11\%$), 2,2,2-trichloroethanol (by $23 \pm 6\%$), isoflurane (by $32 \pm 10\%$), AA29504 (by $41 \pm 6\%$), loreclezole (by $43 \pm 9\%$), but significantly increased those potentiated by muscimol (by $26 \pm 11\%$). Naphthalene-etomidate significantly increased currents potentiated by a low (1 μ M) diazepam concentration (by $56 \pm 14\%$) while reducing those potentiated by a high (100 μ M) diazepam concentration (by $11 \pm 7\%$).

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Conclusions—Our results suggest that many (but not all) sedative-hypnotics are capable of positively modulating the GABA_A receptor by binding within a common set of hydrophobic cavities.

Keywords

Competitive antagonist; GABA_A receptor; Sedative-hypnotic

Introduction

Many sedative-hypnotics produce their therapeutic actions – at least in part – by positively modulating (“potentiating”) the function of γ -aminobutyric acid type A (GABA_A) receptors. Consequently, there has been an intense effort to determine where on this receptor these drugs bind and act in order to better understand the mechanisms that account for sedative-hypnotic action and to guide the development of novel drugs that target this receptor. Such efforts have included the use of x-ray crystallography to define where sedative-hypnotics bind on GABA_A receptor-like proteins along with photoaffinity labeling, site-directed mutagenesis, cryo-electron microscopy, and substituted cysteine modification protection studies to identify the amino acids in the GABA_A receptor that line the binding cavity. [1–6] Each of these techniques has important limitations. However together, they have allowed the identification of numerous cavities within the GABA_A receptor where sedative-hypnotics may bind (figure 1A & B). [7] These include two distinct but homologous sets of hydrophobic cavities located within the receptor’s transmembrane domain at the two β^+ – α^- subunit interfaces and the α^+ – β^- and γ^+ – β^- subunit interfaces. [3, 8] Photoaffinity label protection studies indicate that etomidate binds selectively within the cavities located at the two β^+ – α^- subunit interfaces, pentobarbital binds selectively within those located at the α^+ – β^- and γ^+ – β^- subunit interfaces, whereas propofol binds to all four of these inter-subunit transmembrane cavities relatively non-selectively. [3] Cavities where sedative-hypnotics (i.e. propofol) bind have also been located between subunits within the receptor’s extracellular domain and between its extracellular and transmembrane domains, and the existence of *intra*-subunit binding cavities has also been suggested for alcohols and volatile anesthetics. [1, 7] Because the GABA_A receptor contains many solvent-accessible cavities which may be capable of binding sedative-hypnotic agents and given the inherent limitations of available techniques, it has often been difficult to establish for any individual drug which of these cavities mediate positive receptor modulation. Thus, multiple approaches whose results converge will likely be necessary to confidently locate them.

One way to test whether the binding of a sedative-hypnotic to a specific receptor site produces positive modulation is to determine whether such modulation can be reversed by the addition of a lower efficacy ligand that also binds to that site (i.e. competitive antagonism). We recently developed a ligand (naphthalene-etomidate) that binds within both classes of inter-subunit transmembrane cavities of the GABA_A receptor but possesses relatively low positive modulatory efficacy. [9] Consequently, it acts as a competitive antagonist capable of reversing the positive modulatory actions of more efficacious ligands that act by binding within these cavities (e.g. propofol, etomidate, and pentobarbital). Conversely, it allosterically enhances the actions of ligands that act via other GABA_A

receptor sites because it is not completely without positive modulatory efficacy. [9] In this report, we describe studies that utilized this compound as a pharmacological screening tool to test whether seven chemically diverse clinical and experimental sedative-hypnotics can positively modulate the GABA_A receptor by binding within these inter-subunit transmembrane cavities.

Materials and Methods

All studies were conducted with the approval of and in accordance with rules and regulations of the Institutional Animal Care and Use Committee at the Massachusetts General Hospital, Boston, Massachusetts, the principles outlined in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health, and in compliance with ARRIVE guidelines. Adult female *Xenopus laevis* frogs were purchased from Xenopus One (Ann Arbor, MI). Naphthalene-etomidate was synthesized by Aberjona Laboratories (Beverly, MA) as previously described. [9] Isoflurane solutions were prepared and delivered using Teflon tubing from gas-tight syringes as dilutions from saturated stocks assuming a saturated solubility of 15 mM in buffer. [10] All other sedative-hypnotics were prepared as dilutions from stock solutions prepared in buffer or dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was always <0.1%, which pilot studies showed does not affect GABA_A receptor currents.

GABA_A Receptor Direct Activation Assay

Oocytes (stage 4 and 5) were obtained and injected with messenger RNA encoding the α_1 , β_3 , and γ_2 subunits of the human GABA_A receptor. After injection, oocytes were incubated for at least 12 hours at 18°C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH=7.4) containing 0.05 mg/mL of gentamicin before electrophysiological study. All electrophysiological recordings were performed at room temperature using the whole cell two-electrode voltage-clamp technique. [9]

A GABA concentration-current response curve was first generated for each oocyte to define the GABA concentration that elicits 5% of the current evoked by 1 mM GABA (i.e. EC₅ GABA). The oocyte was then perfused for 90 s with EC₅ GABA plus the desired sedative-hypnotic at a concentration that (together with EC₅ GABA) elicits approximately 50% of the current elicited by 1 mM GABA alone. Under these experimental conditions, the magnitude of current potentiation (and thus degree of receptor activation) was similar for all sedative-hypnotics. This sedative-hypnotic concentration was defined in pilot experiments as 3 μ M muscimol, 2 μ M alphaxalone, 2 mM 2,2,2-trichloroethanol, 1.5 mM isoflurane, 1 μ M AA29504, and 10 μ M loreclezole. Because even saturated aqueous concentrations of diazepam cannot potentiate to 50%, it was assessed at concentrations of 1 μ M and 100 μ M, the former representing a concentration that produces flumazenil-reversible potentiation and the latter a concentration that produces flumazenil-*irreversible* potentiation. [11] Thirty seconds into the 90 s activation period, naphthalene-etomidate was added for 30 s. In our initial studies, we utilized a near aqueous saturating naphthalene-etomidate concentration (300 μ M) to maximize the magnitude of any competition with the sedative-hypnotic agent. Subsequent studies used ranging naphthalene-etomidate concentrations to define the

naphthalene-etomidate concentration-dependency of actions on currents potentiated by two representative sedative-hypnotics (muscimol and loreclezole). The effect of naphthalene-etomidate on currents was quantified as the maximum change in current amplitude recorded during naphthalene-etomidate administration as previously described. [9] Between electrophysiological recordings, oocytes were perfused with buffer for at least 3 min to remove GABA and/or drugs and to allow receptors to recover from desensitization.

Statistical Analysis

All data are reported as either the within-sample change derived from a single oocyte experiment or the mean $\pm$ SD of the within-sample change derived from six separate oocyte experiments. In addition, 95% confidence intervals for the means are reported in table 1. For each sedative-hypnotic, a one-sample t test (two tailed) was used to statistically test whether the current amplitude evoked by EC₅ GABA and potentiated by the sedative-hypnotic was significantly altered upon the addition of 300 μ M naphthalene-etomidate. The false discovery rate (FDR) for these tests was controlled using the Benjamini-Hochberg approach. Discovery was defined as a q-value < 0.05 . Our sample size was chosen based on our previous experience and expected to provide a 90% power to detect a 20% change in current amplitude upon addition of naphthalene-etomidate. This change approximates those previously seen in studies using the sedative-hypnotics etomidate and pentobarbital. [9] The potency of naphthalene-etomidate for modifying EC₅ GABA currents potentiated by muscimol and loreclezole was quantified as either an EC₅₀ (for muscimol) or IC₅₀ (for loreclezole) using a three parameter Hill equation. The uncertainties in the calculated EC₅₀ and IC₅₀ values are given as 95% confidence intervals (95% CI). All statistical analyses were performed using Prism 8 for Mac OS X (Graphpad Software, La Jolla, CA).

Results

Figure 2A shows representative electrophysiological traces demonstrating the varied impact of 300 μ M naphthalene-etomidate on GABA_A receptor-mediated currents that were elicited with EC₅ GABA and potentiated by each of the 7 sedative-hypnotics. Naphthalene-etomidate reduced the amplitudes of currents that were potentiated by alphaxalone, 2,2,2-trichloroethanol, isoflurane, AA29504, and loreclezole, but increased those potentiated by muscimol. Naphthalene-etomidate's effect on currents potentiated by diazepam were dependent upon the diazepam concentration used as it increased the amplitude of currents potentiated by a low diazepam concentration (1 μ M) but reduced those potentiated by a high diazepam concentration (100 μ M).

The mean changes in current amplitudes produced by naphthalene-etomidate on EC₅ GABA currents potentiated by the 7 sedative-hypnotics are plotted in figure 2B and reported in table 1. Naphthalene-etomidate significantly reduced currents potentiated by (mean of 6 separate experiments $\pm$ SD) alphaxalone (by $22 \pm 11\%$), 2,2,2-trichloroethanol (by $23 \pm 6\%$), isoflurane (by $32 \pm 10\%$), AA29504 (by $41 \pm 6\%$), loreclezole (by $43 \pm 9\%$), and 100 μ M diazepam (by $11 \pm 7\%$), but significantly increased those potentiated by muscimol (by $26 \pm 11\%$) and 1 μ M diazepam (by $56 \pm 14\%$).

Figure 3 plots the mean change in current amplitude produced by a range of naphthalene-etomidate concentrations on EC₅ GABA currents potentiated by two representative sedative-hypnotics: muscimol and loreclezole. Naphthalene-etomidate modified the amplitudes of currents potentiated by both sedative-hypnotics in a concentration-dependent manner. It increased currents potentiated by muscimol with an EC₅₀ of 3.9 μ M (95 %CI: 0.9 – 17 μ M) and decreased currents potentiated by loreclezole with an IC₅₀ of 76 μ M (95% CI: 60 – 97 μ M).

Discussion

Our goal in undertaking these studies was to test the hypothesis that many chemically diverse clinical and experimental sedative-hypnotic drugs are capable of potentiating GABA_A receptor function by binding within the same homologous set of cavities that bind propofol, etomidate, and barbiturates, and are located within the receptor's transmembrane domain between the two β^+ – α^- subunits (propofol and etomidate) and its α^+ – β^- and γ^+ – β^- subunits (propofol and pentobarbital). Our experimental approach was to determine whether naphthalene-etomidate – an etomidate analog that binds within these inter-subunit transmembrane cavities but possesses relatively low intrinsic modulatory efficacy – could reverse such potentiation. We interpret such a finding as evidence supporting a competitive interaction between naphthalene-etomidate and the sedative-hypnotic agent for binding within either (or both) classes of cavities. Conversely, we interpret enhanced potentiation by naphthalene-etomidate as indicating that the sedative-hypnotic acts via a different class(es) of sites. Under these circumstances, the weak positive modulatory activity of naphthalene-etomidate allosterically enhances the potentiation produced by the sedative-hypnotic agent.

Our finding that naphthalene-etomidate reduced currents that were potentiated by alphaxalone, 2,2,2-trichloroethanol, isoflurane, AA29504, and loreclezole lends support to our hypothesis and is consistent with previous studies suggesting that all of these drugs (or short and long chain alcohols in the case of 2,2,2-trichloroethanol) are capable of binding within these inter-subunit transmembrane cavities (Table 1). [2, 12–17] In contrast to its inhibitory effect on currents potentiated by the sedative-hypnotics listed above, we observed that naphthalene-etomidate increased currents that were potentiated by muscimol, a GABA analog with sedative-hypnotic properties that presumably acts via the agonist binding sites located in the extracellular domain.[18] The differential effects of naphthalene-etomidate on currents that were potentiated by 1 μ M versus 100 μ M diazepam is most simply attributed to the two distinct, concentration-dependent mechanisms by which the benzodiazepine can potentiate GABA_A receptors. [11] At low concentrations, diazepam acts in a flumazenil-reversible manner by binding with high affinity to the classical benzodiazepine site located in the receptor's extracellular domain. [19] Under these conditions (and similar to our results using muscimol), naphthalene-etomidate allosterically increased diazepam-potentiated currents because the two compounds were binding within different cavities (located in the transmembrane and extracellular domains, respectively). However, at a high concentration where diazepam potentiates in a flumazenil-irreversible manner by also binding to a low affinity site, naphthalene-etomidate reduced diazepam-potentiated currents suggesting competition for binding within the transmembrane inter-subunit cavities. This interpretation would explain why mutating amino acids that line these cavities (and abolish sensitivity to

etomidate) also abolish sensitivity to high (but not low) concentrations of diazepam: At high concentrations, diazepam can potentiate by binding within the same inter-subunit transmembrane cavities on the GABA_A receptor as etomidate. [11] Such an interpretation is also consistent with recent cryo-electron microscopic studies demonstrating that diazepam can bind within two distinct classes of $\alpha_1\beta_3\gamma_{2L}$ GABA_A receptor cavities, one located in the extracellular domain at the $\alpha^+-\gamma^-$ subunit interface (i.e. the classical high affinity benzodiazepine binding site) and the other located in the transmembrane domain at the two $\beta^+-\alpha^-$ subunit interfaces (i.e. the etomidate binding site).

One limitation of this study is that we cannot formally rule out non-competitive interactions (i.e. negative allosterism) to explain the ability of naphthalene-etomidate to reduce the positive modulatory actions of certain sedative-hypnotics. However, co-agonist models of GABA_A receptor function obviate the possibility of such interactions between two positive modulators (albeit an intrinsically weak one in the case of naphthalene-etomidate); within the context of such models, the magnitude of potentiation produced by two positive allosteric modulators acting at separate sites cannot be less than that produced by either alone. [20]

In summary, naphthalene-etomidate reverses the positive modulatory actions of many (but not all) sedative-hypnotic agents. While not eliminating the possibility that other GABA_A receptor cavities may also be important, our results support the hypothesis that many chemically diverse clinical and experimental sedative-hypnotic drugs can potentiate GABA_A receptor function by binding within the same homologous set of transmembrane inter-subunit cavities that also bind propofol, etomidate, and barbiturates. Thus, binding within these cavities may represent a common, but not universal, mechanism for GABA_A receptor modulation by sedative-hypnotic agents.

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Figure 1:

Schematic representation of the γ -aminobutyric acid type A (GABA_A) receptor viewed as a cross section at the level of either the transmembrane domain (A) or the extracellular domain (B). Solvent-accessible cavities are located at the interfaces between subunits in both the transmembrane and extracellular domains. Each distinct cavity is highlighted by a different color. (A) Two homologous classes of hydrophobic cavities have been defined by photoaffinity labeling studies and are located within the transmembrane domain between receptor subunits. One class of cavities is located at the two $\beta^+ - \alpha^-$ subunit interfaces (highlighted in blue) and the other is located at the $\alpha^+ - \beta^-$ and $\gamma^+ - \beta^-$ subunit interfaces (highlighted in yellow and purple, respectively). Etomidate binds selectively to the former class of cavities whereas pentobarbital binds selectively to the latter. Propofol and naphthalene-etomidate bind relatively non-selectively to both classes of cavities. Naphthalene-etomidate possesses low intrinsic positive modulatory efficacy and thus can act as a competitive antagonist for more efficacious sedative-hypnotics that bind within these cavities. (B) Additional cavities are located in the extracellular domain. These cavities do not bind naphthalene-etomidate. The extracellular cavities located at the two $\beta^+ - \alpha^-$ subunit interfaces (highlighted in red) bind orthosteric agonists (e.g. GABA and presumably muscimol) and the extracellular cavity located at the $\alpha^+ - \gamma^-$ subunit interface (highlighted in green) binds benzodiazepines (e.g. diazepam) with high affinity. Each subunit has an interface with two other subunits, defining a “+” and “-” side to each subunit.

Figure 2:

Differential modulation of sedative-hypnotic-potentiated GABA_A γ -aminobutyric acid type A (GABA_A) receptor currents by naphthalene-etomidate. (A) Representative current traces obtained upon receptor activation for 90 s with GABA at a concentration that evokes 5% of the current evoked by 1 mM GABA (EC₅ GABA) along with the indicated sedative-hypnotic agent. Thirty seconds into this activation period, 300 μ M naphthalene-etomidate was added for 30 s. The gray bars highlight the time periods when naphthalene-etomidate was applied. The dotted black line shows the interpolated line used as the baseline against which the effect of naphthalene-etomidate was quantified. The red and green arrows indicate the respective decrease and increase in current amplitude produced by naphthalene-etomidate. To facilitate visual comparisons, all current amplitudes were normalized to the peak current produced by EC₅ GABA plus sedative-hypnotic prior to the addition of naphthalene-etomidate. This amplitude is indicated by the solid blue line. (B) Percent change in current amplitude produced by the addition of 300 μ M naphthalene-etomidate to EC₅ GABA-elicited currents potentiated by the indicated sedative-hypnotics. Positive values indicate that naphthalene-etomidate increased current amplitudes whereas negative values indicate that it reduced current amplitudes. Each symbol represents data from an individual oocyte experiment. Mean $\pm$ SD are also indicated for each dataset. The EC₅ GABA concentration was defined for each individual oocyte and averaged 6.3 ± 2.0 μ M. The false discovery rate

(FDR) for these tests was controlled using the Benjamini-Hochberg approach. * $q < 0.05$; ** $q < 0.01$; *** $q < 0.001$.

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Figure 3:

Concentration-dependent effects of naphthalene-etomidate on EC_5 GABA currents potentiated by either muscimol or loreclezole. Naphthalene-etomidate increased currents potentiated by muscimol with an EC_{50} of $3.9 \mu\text{M}$ (95 %CI: $0.9 - 17 \mu\text{M}$) and decreased currents potentiated by loreclezole with an IC_{50} of $76 \mu\text{M}$ (95% CI: $60 - 97 \mu\text{M}$). The amplitudes of the muscimol-potentiated and loreclezole-potentiated currents in the absence of naphthalene-etomidate were constrained to a value of 100%. At infinite concentrations, naphthalene-etomidate potentiates the peak amplitude of EC_5 GABA-evoked currents to a value that is 11% of that evoked by 1 mM GABA. [9] Therefore, the amplitude of loreclezole-potentiated currents in the presence of infinite concentrations of naphthalene-etomidate was constrained to a value of 11%, implying complete displacement of loreclezole by naphthalene-etomidate. The EC_5 GABA concentration was defined for each individual oocyte and averaged $7.0 \pm 1.6 \mu\text{M}$ for the muscimol studies and $5.4 \pm 1.5 \mu\text{M}$ for the loreclezole studies. Each symbol represents the mean $\pm$ SD of six experiments using different oocytes.

Table 1:

Inhibition or Potentiation of Positively Modulated γ -Aminobutyric Acid Type A (GABA_A) Receptor-Mediated Currents by 300 μ M Naphthalene-Etomidate

Compound Name	Molecular Structure	Current Amplitude Change $\pm$ SD (95% Confidence Interval) % 1 mM GABA	Receptor Domains Containing Putative Interactions Sites*
Muscimol		26 ± 11 (14 to 37)	Extracellular domain, inter-subunit [18]
Alphaxalone		-22 ± 11 (-11 to -33)	Transmembrane domain, inter-subunit [12, 16] Transmembrane domain, intra-subunit [16]
2,2,2-Trichloroethanol		-23 ± 6 (-17 to -29)	Transmembrane domain, inter-subunit [6, 15] Transmembrane domain, intra-subunit [1]
Isoflurane		-32 ± 10 (-22 to -43)	Transmembrane domain, inter-subunit [6, 15] Transmembrane domain, intra-subunit [1]
AA29504		-41 ± 6 (-35 to -46)	Transmembrane domain, inter-subunit [13]
Loreclezole		$-43 \pm 9\%$ (-34 to -52)	Transmembrane domain, inter-subunit [14, 17]
Diazepam		$56 \pm 14\%$ (1 μ M) (41 to 70)	Extracellular domain, inter-subunit [4,19]
		$-11 \pm 7\%$ (100 μ M) (-3 to -18)	Transmembrane domain, inter-subunit [4, 11]

* GABA_A receptor (or receptor-like) domains containing putative interaction sites for the indicated sedative-hypnotic (or one from the same chemical class).