Additive Effects of Sevoflurane and Propofol on \(\gamma\)-Aminobutyric Acid Receptor Function

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Background: Previous studies have shown that propofol and sevoflurane enhance the function of \(\gamma\)-aminobutyric acid type A (GABA\(_A\)) receptors. However, it is not known whether these two drugs modulate the same molecular pathways. In addition, little is known about receptor function in the presence of both propofol and sevoflurane. The aim of this study was to better understand the interactions of propofol and sevoflurane with the GABA\(_A\) receptor.

Methods: Wild-type \(\alpha_1, \beta_2, \gamma_2\) GABA\(_A\) receptor subunit complementary DNAs were transfected into human embryonic kidney cells grown on glass coverslips using a calcium phosphate transfection method. After transfection (36–72 h), cells were whole cell patch clamped and exposed to combinations of the following: 0.3–1,000 \(\mu\)M \(\gamma\)-aminobutyric acid (GABA), 0–10 \(\mu\)M propofol, and 0–1,650 \(\mu\)M sevoflurane. Chemicals were delivered to the cells using two 10-channel infusion pumps and a rapid solution exchanger.

Results: Both propofol and sevoflurane alone enhanced the amplitude of GABA\(_A\) receptor responses to submaximal concentrations of GABA in a dose-dependent manner. The enhancement was underpinned by an increase in the apparent affinity of the receptor for GABA. Coapplication of both anesthetics further enhanced the apparent affinity of the receptor for GABA.

Conclusions: Response surface modeling of the potentiation of GABA responses (0.3–1,000 \(\mu\)M) by sevoflurane and propofol revealed that the two anesthetics modulated receptor function in an additive manner. These results are consistent with recent mutagenesis studies, suggesting that these two drugs have separate binding sites and converging pathways of action on the GABA\(_A\) receptor.

The aim of this study and an accompanying clinical study is to better understand the interactions of propofol and sevoflurane.

Anesthetic interactions fall into three categories: The drugs can be additive, antagonistic (subadditive), or synergistic (supra-additive). Additive interactions occur when the effect from two drugs in combination equals the effect of either alone in an amount equal to the sum of the two drugs, after normalizing the concentrations to the intrinsic potency of each drug. Subadditive interactions occur when the effect of the combination is less than either alone, and synergism occurs when the effect is greater than either alone.¹ Additive interactions often occur when two drugs act via a similar mechanism, whereas synergism occurs when the two drugs act via different mechanisms.² Anesthesiologists have become experts at manipulating multidrug regimens. By making use of synergistic drug combinations to achieve a desired end point in an anesthetic-sparing manner, it is the hope of anesthesiologists to reduce patient recovery time and to increase patient safety.

Propofol and sevoflurane have both been shown to enhance the function of the \(\gamma\)-aminobutyric acid type A (GABA\(_A\)) receptor in neurons and in recombinant systems.³–⁶ GABA\(_A\) receptors mediate the fast inhibition of neuronal excitability by \(\gamma\)-aminobutyric acid (GABA), the most common inhibitory neurotransmitter in the central nervous system. GABA\(_A\) receptors are ligand-gated ion channels constructed from five subunits. The \(\alpha_1\beta_2\gamma_2\) receptor subtype is the most prevalent in synapses of the adult mammalian central nervous system, accounting for approximately 40% of the total complement of GABA\(_A\) receptors.⁷,⁸ The function of neuronal GABA\(_A\) receptors is modulated by many general anesthetics⁹ at concentrations consistent with their clinical use.¹⁰

Recent site-directed mutagenesis studies have provided compelling evidence that many general anesthetics interact with two distinct binding sites on the GABA\(_A\) receptor. Mutation of a conserved serine (Ser270) in the second transmembrane domain of the \(\alpha\) subunit is sufficient to block receptor enhancement by halogenated ether and alkane anesthetics such as isoflurane, sevoflurane, halothane, and chloroform.¹¹–¹⁴ Conversely, mutation of a conserved asparagine (Asn265) in the second transmembrane domain or a conserved methionine (Met286) in the third transmembrane domain of the \(\beta\) subunit abolishes receptor modulation by propofol and etomidate.¹⁵,¹⁶ It is not yet known how drug interactions at these loci result in an enhancement of receptor func-

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tion. The aim of this study was to determine whether receptor modulation by combinations of both anesthetics occurred in an additive, synergistic, or antagonistic manner.

Materials and Methods

γ-Aminobutyric acid type A receptor complementary DNAs (cDNAs; gift from Neil Harrison, Ph.D., Professor of Pharmacology and Director, C.V. Starr Laboratory of Molecular Neuropharmacology, Department of Anesthesiology, Weill Cornell Medical College, New York, New York) were expressed via the vector pCIS2 in human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) as previously described.17 HEK-293 cells were cultured on poly-D-lysine–treated coverslips in a solution containing Eagle minimum essential medium supplemented with 5% fetal bovine serum and 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 100 μg/ml gentamicin. For the transient expression of GABA A receptors, cells were transfected using the CaPO 4 precipitation technique.18,19 The GABA A receptor cDNAs and adeno-associated virus–green fluorescent protein cDNA (gift from H. Trent Spencer, Ph.D., Assistant Professor, Department of Pediatrics, Division of Hematology/Oncology and Bone Marrow Transplantation, Emory University School of Medicine, Atlanta, Georgia) were precipitated for 30 min at room temperature in a 160-μl solution containing 125 mM CaCl2, 140 mM NaCl, 750 μM Na2HPO4, and 2.5 μg of each cDNA. After 30 min, the mixture was added to cells grown on coverslips. The cDNA was in contact with the HEK cells for 24 h in an atmosphere containing 3% CO2 (37°C) before being removed and replaced with fresh culture medium in an atmosphere of 5% CO2 (37°C).

Coverslips of transfected cells were transferred 48–72 h after cDNA removal to a recording chamber and superfused with extracellular saline before application of one of eight GABA concentrations for 2 s, followed by a return to saline for at least 8 s before any subsequent GABA application. Below 100 μM GABA, the responses did not desensitize; at and above 100 μM, the amplitude of the responses declined by 10–15% in the continued presence of the agonist. Responses were low-pass-filtered (100 Hz; –3 dB, four-pole Bessel) and digitized with a 1322A interface (Axon Instruments) using pCLAMP 9 and stored for off-line analysis. Because intracellular and extracellular solutions contained equal chloride concentrations (145 mM), the chloride equilibrium potential was around 0 mV. All experiments were performed at room temperature (21°–24°C).

Stock solutions of GABA and propofol were diluted in extracellular solutions shortly before use. Sevoflurane solutions were prepared by injection of liquid anesthetic with a gas-tight syringe (Hamilton, Reno, NV) into intravenous drip bags containing defined volumes of extracellular solutions (100 ml) and used for up to 4 h. Clinically relevant concentrations of general anesthetics were used throughout the study; the aqueous concentration for 1 minimum alveolar concentration (MAC) sevoflurane was taken to be 330 μM,5 and the anesthetic EC50 (AC50) concentration for propofol was taken to be 2 μM.21 Losses of general anesthetics in this perfusion system have been measured using gas chromatography and typically represent only 5–10% of the initial total drug concentration.22 Sevoflurane was obtained from Abbott Laboratories (North Chicago, IL), and propofol (2,6 di-isopropylphenol) was obtained from Sigma (St. Louis, MO).

For each GABA exposure, the peak current amplitudes were measured and the GABA concentration–response data for each cell with and without general anesthetic were extracted from the raw data using our own software package. The analysis software was written to calculate nonlinear dose response curve parameters using Visual Basic macros within Microsoft Excel (Microsoft Corp., Redmond, WA) to facilitate efficient data organization. Dose–response parameters were optimized using GRG2, a version of the Generalized Reduction Gradient algorithm included in Microsoft Excel.23 Using iterative processing and extensively automated file handling, we were able to process multiple data streams simultaneously.

The current peaks were fitted to a Hill equation of the form I = I max * [GABA] nH/([GABA] nH + EC 50 nH), where I is the peak of each current, I max is the maximum

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whole-cell current amplitude, \([\text{GABA}]\) is the GABA concentration, \(EC_{50}\) is the GABA concentration eliciting a half-maximal current, and \(n_H\) is the Hill coefficient.

Concentration–response relations were recorded in the absence and presence of general anesthetic in the same cell. This enabled us to determine the control and anesthetic-modulated GABA EC_{50}s, defined as \(EG_{50}\) and \(EO_{50}\)' respectively. The fractional effect on \(EC_{50}\) was defined as \((1 - EG_{50}/EO_{50})\).

Concentration–effect relations were calculated for propofol and sevoflurane. The fractional effects of anesthetic alone on GABA \(EC_{50}\) were fitted to a Hill equation of the form

\[ E = \frac{\text{anesthetic}^{slope}/(\text{anesthetic}^{slope} + C_{50}^{slope}), \] where \(E\) is the fractional effect of the anesthetic on GABA \(EC_{50}\). For sinistral concentration–response shifts (0 < \(E\) ≤ 1), \([\text{anesthetic}]\) is the concentration of propofol or sevoflurane, \(C_{50}\) is the anesthetic concentration eliciting a half maximal effect, and slope is the Hill coefficient for the anesthetic concentration–effect relation. Statistical significance was assessed using a one-way analysis of variance with Dunnett test for multiple comparisons. Data are presented as mean ± SEM.

The response surface for the modulation of GABA \(_{A}\) receptor function by propofol and sevoflurane was determined using the method of Minto et al. Briefly, anesthetic concentrations were normalized to the \(C_{50}\) of each drug:

\[ U_A = \frac{[A]}{C_{50,A}}, \]

\[ U_B = \frac{[B]}{C_{50,B}}. \]

We used \(U_A\) and \(U_B\) to define a new variable \(\theta\), the drug ratio of \(A\) and \(B\):

\[ \theta = \frac{U_B}{U_A + U_B}. \]

In the absence of sevoflurane (propofol alone), \(U_A = 0\) and hence \(\theta = 1\). Conversely, in the absence of propofol (sevoflurane alone), \(U_B = 0\) and hence \(\theta = 0\). When equal quantities of the drugs are present, \(\theta = 0.5\).

Substituting these terms into the Hill equation, we obtain the following function:

\[ E = \frac{U_A + U_B \gamma(\theta)}{1 + (U_A + U_B \gamma(\theta))}. \]

\(U_{50}(\theta)\) defines the potency of the drug combination relative to the potency of either drug alone, and \(\gamma(\theta)\) is the sigmoidicity of the response surface. When \(U_{50}(\theta) > 1\), the interaction is synergistic. When \(U_{50}(\theta) < 1\), the interaction is subadditive or antagonistic. When \(U_{50}(\theta) = 1\), the interaction is additive.

It has been demonstrated that isoboles have a simple inward or outward curvature and that they can be well approximated by a simple second order polynomial of the form

\[ U_{50}(\theta) = 1 - \beta_{21}\theta + \beta_{22}\theta^2 \]

and

\[ \gamma(\theta) = 1 - \beta_{21}\theta + \beta_{22}\theta^2. \]

These functions were fitted to the concentration–effect data using the Gauss–Newton nonlinear least-squares method (Statistics Toolbox function “nlinfit”; MATLAB, Natick, MA). The results were verified using the NONMEM program (Globomax, Hanover, MD).

**Results**

After transfection with adeno-associated virus–green fluorescent protein and GABA\(_{A}\) receptor \(\alpha_1, \beta_2,\) and \(\gamma_2\) and cDNAs, HEK-293, more than 90% of the cells were found to fluoresce, indicating that successful transfection conditions had occurred. Fluorescing cells were whole cell voltage clamped at −60 mV and superfused with extracellular saline. Application of GABA at eight different concentrations (0.3–1,000 \(\mu\)M) to the cells under these conditions elicited inward chloride currents in a concentration-dependent manner. Addition of 2 \(\mu\)M propofol to the extracellular medium resulted in an increase in the amplitudes of currents activated by 1–30 \(\mu\)M GABA, a small decrease in peak currents activated by 100–1,000 \(\mu\)M GABA, and an increase in the baseline noise of the recording. A representative whole cell voltage clamp recording showing the effect of 2 \(\mu\)M propofol on GABA\(_{A}\) receptor function is shown in figure 1. These effects were fully reversible. Propofol, 2 \(\mu\)M, reduced the GABA \(EC_{50}\) by a factor of 0.59 ± 0.05, a fractional effect of 0.41 ± 0.05 (table 1). These experiments were repeated using 0.2–10 \(\mu\)M propofol. Propofol was found to reduce the GABA \(EC_{50}\) in a concentration-dependent manner. The \(C_{50}\) for the effect of propofol on the GABA \(EC_{50}\) was \(C_{50,P} = 2.1 ± 0.1 \mu\)M, and the slope was 1.02 ± 0.05.

Next, we repeated these experiments using sevoflurane. Addition of 350 \(\mu\)M sevoflurane to the extracellular saline also resulted in an increase in the amplitudes of currents activated by 1–30 \(\mu\)M GABA and an increase in the baseline noise of the recording (see representative trace in fig. 2). However, in contrast to the experiments with propofol, no reduction in the amplitudes of currents activated by 100–1,000 \(\mu\)M GABA was observed. These effects were also fully reversible. Sevoflurane, 330 \(\mu\)M, reduced the GABA \(EC_{50}\) by a factor of 0.46 ± 0.06, a fractional effect of 0.54 ± 0.06 (table 2). These exper-
The fractional effects of combinations of propofol and sevoflurane on GABA EC50 are shown in table 3. Figure 3 shows a representative recording of the combined effect of 1 μM propofol and 165 μM sevoflurane on currents activated by 0.3–1,000 μM GABA. As was observed with sevoflurane and propofol applied alone, the drug combination resulted in an increase in the amplitudes of currents activated by 1–30 μM GABA, a small decrease in peak currents activated by 100–1,000 μM GABA, and an increase in the baseline noise of the recording. These effects were fully reversible. A combination of 1 μM propofol with 165 μM sevoflurane resulted in a fractional effect of 0.52 ± 0.05 (table 3). The data shown in table 3 was used to construct a response surface model for the modulation of GABA_A receptor function by propofol and sevoflurane (fig. 4).

**Table 1. Fractional Effect of 0–10 μM Propofol on GABA EC50**

<table>
<thead>
<tr>
<th>[Propofol], μM</th>
<th>0</th>
<th>0.2</th>
<th>0.4</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractional effect on GABA EC50</td>
<td>0 ± 0.01</td>
<td>-0.03 ± 0.05</td>
<td>0.06 ± 0.13</td>
<td>0.35 ± 0.02</td>
<td>0.41 ± 0.05</td>
<td>0.5 ± 0.06</td>
<td>0.84 ± 0.01</td>
</tr>
</tbody>
</table>

Values are mean ± SEM and are determinations of at least five concentration-response shifts from at least three cells, as determined by the fractional change in the effective γ-aminobutyric acid concentration for 50% of maximal activation (GABA EC50). Propofol concentrations are reported as molar concentrations. These concentrations equate to 0, 0.1, 0.2, 0.5, 1, 2, and 5 times the anesthetic EC50 of propofol.
and $\beta_{2\gamma}$ were both close to zero. A NONMEM verification revealed that $\beta_{2u} = 0.25 \pm 0.49$ and $\beta_{2v} = 0.16 \pm 0.47$ and were not significantly different from zero. Because neither of the fitted functions significantly deviated from unity, we concluded that neither synergism nor antagonism was occurring and that combinations of propofol and sevoflurane resulted in a purely additive effect on receptor function.

The primary aim of this study and its companion article was to determine whether propofol and sevoflurane had additive or synergistic effects in humans and on human receptors. In humans, synergy with anesthetic drugs is common. Opioids have been shown to reduce general anesthetic requirement (MAC) but are without any intrinsic anesthetic potency when administered alone. Therefore, the interactions between opioids and general anesthetics can be considered to be highly synergistic. A similar argument can be made for the synergistic actions of midazolam and thiopental. Propofol and sevoflurane, however, are both general anesthetics in their own right, and we might therefore expect there to be less synergism between them. In the companion study to this report, Harris et al. have shown that in humans, there is indeed no synergism between propofol and sevoflurane for immobility and loss of consciousness. Instead, the two anesthetics were shown to be additive. In this study, we have also shown that these two general anesthetics are additive in their actions on GABA$_A$ receptors, the most common fast inhibitory neurotransmitter receptor in both the brain and the spinal cord, sites thought to be critical for loss of consciousness and immobility, respectively.

The two drugs used in this study were selected for three important reasons. First, although both sevoflurane and propofol can be used alone for both induction and maintenance of anesthesia, these two anesthetics are commonly used together in the clinical setting. Second, these two anesthetics are chemically very different; sevoflurane is a small fluorinated ether, whereas propofol is a large phenol, and it is therefore unlikely that both compounds are capable of making the same array of bonds within a common anesthetic binding site. Finally, both propofol and sevoflurane exist as a single optical isomer. Conducting an additivity study with a pair of anesthetics, one of which exists as a racemic mixture of two or more stereoisomers, would have required an initial investigation into the synergistic, additive, or antagonistic activity of each of the isomers with respect to the other, before any consideration of the second drug could be undertaken. By using propofol and sevoflurane, we removed this requirement from our study.

We hypothesized that it would be unlikely for propofol and sevoflurane to compete for the same binding within the GABA$_A$ receptor because of the large differences in their molecular structure. This hypothesis is strongly supported by the results of site directed mutagenesis experiments that are consistent with the hypothesis that inhaled general anesthetics mediate their effects within

### Table 2. Fractional Effect of 0–1.65 mM Sevoflurane on GABA EC$_{50}$

<table>
<thead>
<tr>
<th>[Sevoflurane], $\mu$m</th>
<th>0</th>
<th>33</th>
<th>66</th>
<th>165</th>
<th>330</th>
<th>660</th>
<th>1,650</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractional effect on GABA EC$_{50}$</td>
<td>0 $\pm$ 0.01</td>
<td>0.08 $\pm$ 0.01</td>
<td>0.11 $\pm$ 0.11</td>
<td>0.36 $\pm$ 0.01</td>
<td>0.54 $\pm$ 0.06</td>
<td>0.71 $\pm$ 0.07</td>
<td>0.81 $\pm$ 0.02</td>
</tr>
</tbody>
</table>

Values are mean $\pm$ SEM and are determinations of at least five concentration-response shifts from at least three cells, as determined by the fractional change in the effective $\gamma$-aminobutyric acid concentration for 50% of maximal activation (GABA EC$_{50}$). Sevoflurane concentrations are reported as molar concentrations. These concentrations equate to 0, 0.1, 0.2, 0.5, 1, 2, and 5 times sevoflurane minimum alveolar concentration.

### Table 3. Fractional Effect of Propofol and Sevoflurane Combinations on GABA EC$_{50}$

<table>
<thead>
<tr>
<th>[Propofol], $\mu$m</th>
</tr>
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<tbody>
<tr>
<td>[Sevoflurane], $\mu$m</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>33</td>
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<tr>
<td>66</td>
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<tr>
<td>165</td>
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<tr>
<td>330</td>
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<tr>
<td>660</td>
</tr>
<tr>
<td>1,650</td>
</tr>
</tbody>
</table>

Values are mean $\pm$ SEM and are determinations of at least five concentration-response shifts from at least three cells, as determined by the fractional change in the effective $\gamma$-aminobutyric acid concentration for 50% of maximal activation (GABA EC$_{50}$). Sevoflurane concentrations are reported as molar concentrations. These concentrations equate to 0, 0.1, 0.2, 0.5, 1, 2, and 5 times sevoflurane minimum alveolar concentration.
Fig. 3. When applied together, 1 μM propofol and 165 μM sevoflurane enhance currents activated by low (< 30 μM) concentrations of γ-aminobutyric acid (GABA). Whole cell responses to 0.3, 1, 3, 10, 30, 100, 300, and 1,000 μM GABA in the absence (first eight responses) and presence (last eight responses) of 1 μM propofol and 165 μM sevoflurane. Whole cell recordings were made from HEK-293 cells voltage clamped at -60 mV expressing α1β2γ2s GABA<sub>A</sub> receptor subunits. The filled bar above the current traces denotes the period of propofol and sevoflurane application, and the open bars denote the period of GABA application. The application of 1 μM propofol and 165 μM sevoflurane together shifted the EC<sub>50</sub> for GABA from 25.3 to 15.4 μM, a fractional shift of 0.61.

Fig. 4. Concentration–response surface for the effect of 0–10 μM propofol and 0–165 mM sevoflurane on the fractional change in the effective γ-aminobutyric acid concentration for 50% of maximal activation (GABA EC<sub>50</sub>) for α1β2γ2s GABA<sub>A</sub> receptors expressed in HEK-293 cells. The horizontal axes represent anesthetic concentrations normalized to their EC<sub>50</sub>s for GABA EC<sub>50</sub> modulation (U<sub>α</sub>: sevoflurane; U<sub>β</sub>: propofol). The vertical axis represents the fractional effect on GABA EC<sub>50</sub>. The surface was fitted to the GABA EC<sub>50</sub> modulation data in table 3 using a least squares algorithm written in Matlab.
In this study, we examined the combined effect of clinically relevant concentrations of sevoflurane and propofol on the function of GABA_A receptors containing only the α1, β2, and γ2s receptors. Although this receptor combination accounts for approximately 40% of the GABA_A receptors in the central nervous system, there are several other important tissue-specific combinations that have different kinetics and different general anesthetic sensitivities. It would be interesting if future studies investigated the effects of these two drugs on other subunit combinations (e.g., α2, β3, and γ2) using the techniques described here and also electrophysiologic methods with higher temporal resolutions, e.g., single channel recording.

In conclusion, the data presented in this study and the results in the companion article25 show that propofol and sevoflurane modulate GABA_A receptor function and generate the anesthetized state (immobility and loss of consciousness) in an additive manner. Neither of the two studies detected a significant degree of synergism or antagonism between propofol or sevoflurane in the three assays. Therefore, it seems that the most likely explanation for the results described here is that propofol and sevoflurane enhance GABA_A receptor function, in both the brain and the spinal cord. The two drugs do this by interacting at distinctly different binding sites within the same protein. However, the binding of anesthetic to either of the modulatory sites converges on a single effect: the enhancement of GABA_A receptor gating, resulting in an increase in the open probability of the integral ion channel. The resulting alteration in coincidence detection and synchrony in neuronal networks containing these receptors40,41 is likely to be fundamental in the generation of the anesthetized state.

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