COMPETITIVE INTERACTIONS BETWEEN PROPOFOL AND DIAZEPAM: STUDIES IN 
GABA_\text{A} RECEPTORS AND ZEBRAFISH

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Abstract

Although propofol is among the most commonly administered general anesthetics, its mechanism of action is not fully understood. It has been hypothesized that propofol acts via a similar mechanism as etomidate by binding within the GABA\textsubscript{A} receptor transmembrane receptor domain at the two $\beta^+/\alpha^-$ subunit interfaces with resultant positive allosteric modulation. To test this hypothesis, we leveraged the ability of diazepam to bind to those sites and act as a competitive antagonist. We used oocyte-expressed $\alpha_1\beta_3\gamma_2L$ GABA\textsubscript{A} receptors to define the actions of diazepam (± flumazenil) on currents activated or potentiated by propofol and a zebrafish activity assay to define the impact of diazepam and flumazenil on propofol-induced anesthesia. We found that diazepam increased the amplitudes of GABA\textsubscript{A} receptor-mediated currents at nanomolar concentrations but reduced them at micromolar concentrations. The current amplitude changes produced by nanomolar diazepam concentrations were inhibited by flumazenil whereas those produced by micromolar diazepam concentrations were not. Studies of agonist potentiation showed that the micromolar inhibitory action of diazepam was surmountable by high concentrations of propofol and produced a rightward shift in the propofol concentration-response curve characterized by a Schild slope not statistically significantly different from one, consistent with competition between diazepam and propofol. Although micromolar concentrations of diazepam (plus flumazenil) similarly reduced GABA\textsubscript{A} receptor currents modulated by propofol and etomidate, it only reduced the anesthetic actions of etomidate. We conclude that while both propofol and etomidate can modulate GABA\textsubscript{A} receptors by binding to the $\beta^+/\alpha^-$ subunit interfacial sites, propofol-induced anesthesia likely involves additional target sites.
Significance Statement

Although the drug combination of diazepam and flumazenil reverses the GABA_A receptor positive modulatory actions of both propofol and etomidate, it only reverses the in vivo anesthetic actions of etomidate. These results strongly suggest that distinct mechanisms of action account for the anesthetic actions of these two commonly administered anesthetic agents.
**Introduction**

Propofol (2,6-diisopropylphenol) is one of the most widely administered general anesthetic agents. It is used by anesthesia providers to induce and maintain general anesthesia for surgery and, at lower doses, to sedate patients undergoing less invasive medical procedures. Its popularity among clinicians stems from its rapid onset of action, antiemetic activity, and relatively short duration of action (Skues and Prys-Roberts, 1989; Sahinovic et al., 2018). Similar to other sedative-hypnotics (e.g., benzodiazepines, barbiturates, and isoflurane), propofol may also be used as an antiepileptic agent due to its ability to reduce central nervous system excitability (van Gestel et al., 2005; Zhang et al., 2019).

Although much remains to be learned about the underlying mechanisms responsible for the actions of general anesthetic agents, much progress has been made. In the case of propofol, the γ-aminobutyric acid type A (GABA\(_A\)) receptor is thought to play the major role (Jurd et al., 2003; Brohan and Goudra, 2017; Weir et al., 2017). Propofol has two closely related positive allosteric modulatory actions on this receptor (Jones et al., 1995; Sanna et al., 1995; Pistis et al., 1997). At clinically relevant concentrations, propofol enhances (i.e. potentiates) the actions of GABA, shifting the GABA concentration response curve for GABA\(_A\) receptor activation leftward. At higher concentrations, propofol also directly activates GABA\(_A\) receptors even in the absence of agonist. These actions promote an influx of chloride ions into neurons, leading to neuronal hyperpolarization and reduced excitability. Co-agonist models of anesthetic action indicate that anesthetic-mediated agonist potentiation and direct activation are mediated by the same GABA\(_A\) receptor binding site(s) (Rusch et al., 2004; Ruesch et al., 2012; Germann et al., 2019).
Together, site-directed mutagenesis, photoaffinity labeling, and cryo-electron microscopy studies indicate that functionally relevant propofol binding sites are located at the two $\beta^+/\alpha^-$ subunit interfaces within the transmembrane receptor domain (Siegwart et al., 2002; Siegwart et al., 2003; Chiara et al., 2013; Kim et al., 2020). These are the same sites that mediate etomidate action and (at micromolar concentrations) bind diazepam (figure 1), the latter binding characterized by relatively low intrinsic efficacy for modulating receptor function (Walters et al., 2000; Kim et al., 2020). Photoaffinity labeling studies also suggest the presence of propofol binding sites at homologous transmembrane sites located at the $\gamma^+/\beta^-$ and $\alpha^+/\beta^-$ subunit interfaces, and to a separate site located near the transmembrane/extracellular domain interface (Chiara et al., 2013; Yip et al., 2013). Finally, non-GABAergic sites have also been suggested as possible contributors to the anesthetic actions of propofol (Tang and Eckenhoff, 2018).

By leveraging the ability of diazepam to bind at micromolar concentrations to the two $\beta^+/\alpha^-$ subunit interfacial sites and act as a competitive antagonist (i.e. reversal agent), we recently confirmed the critical importance of these sites in mediating both the GABA$_A$ receptor positive modulatory and anesthetic actions of etomidate (McGrath et al., 2020). Specifically, we demonstrated that in the presence of flumazenil to prevent action via the classical high affinity benzodiazepine binding site, diazepam can competitively antagonize GABA$_A$ receptor activation by etomidate and increase by an order of magnitude the etomidate concentration necessary to reduce light-stimulated activity in a zebrafish model of anesthesia. In the present studies, we tested whether propofol acts via the same receptor mechanism by similarly assessing the ability of diazepam to antagonize propofol action on GABA$_A$ receptors and zebrafish activity. Our results reveal that while the positive modulatory receptor actions of propofol – like those of etomidate – are competitively antagonized by diazepam, this is not
associated with an increase in the propofol concentration necessary to reduce light-stimulated zebrafish activity. Our findings strongly suggest that propofol produces hypnosis via mechanisms that are distinct from those of etomidate, likely involving additional target sites beyond the $\beta^+\alpha^-$ subunit interfacial sites of the GABA$_A$ receptor.
Materials and Methods

Sources of Drugs and Chemicals

Buffer reagents were purchased from either Sigma Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA). Flumazenil (AAJ62537-MC) was purchased from VWR International (Radnor, PA). Diazepam (D0899), GABA (A2125), and DMSO (34869) were purchased from Sigma-Aldrich.

Animals

All studies were approved by our Institutional Animal Care and Use Committee. They also followed the principles outlined in the National Institutes of Health (Bethesda, Maryland) Guide for the Care and Use of Laboratory Animals and ARRIVE guidelines. *Xenopus laevis* adult female frogs were purchased from Xenopus One (Ann Arbor, MI, USA) and housed in the Massachusetts General Hospital Center for Comparative Medicine animal care facility. As required for experimental study, zebrafish (*Danio rerio*) embryos were collected from mating adult pairs and maintained as previously described (McGrath et al. 2020).

GABA<sub>A</sub> Receptor Electrophysiology

Oocytes were injected with 0.1 ng/nL of messenger RNA mixtures encoding for human α<sub>1</sub>, β<sub>3</sub>, and γ<sub>2L</sub> GABA<sub>A</sub> receptor subunits (Genbank accession numbers NM_001191119, NM_021912.3, and NM_198904, respectively) at a ratio of 1α:1β:3γ. This messenger RNA was transcribed *in vitro* from complementary DNAs (in PCDNA3.1 vectors), which were confirmed by the Massachusetts General Hospital Molecular Biology Core to agree with published sequences. Oocytes were then incubated at 18°C for 18-48 hours in ND-96 buffer.
(96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH = 7.4) and supplemented with gentamicin (0.05 mg/mL), ciprofloxacin (0.025 mg/mL), and ampicillin (0.1 mg/mL).

Whole-cell two-electrode voltage-clamp electrophysiological studies were performed using a GeneClamp 500B amplifier (Molecular Devices, San Jose, California) with solution application (flow rates: 2 – 4 mL/min) controlled by a VC³ 8 channel valve commander (ALA Scientific Instruments, Farmingdale, NY) as previously described (McGrath et al., 2020). Before further experimental study, each oocyte was exposed to a 10 s application of 1 mM GABA in ND-96 buffer to assess GABA<sub>A</sub> receptor expression. Any oocyte that failed to elicit a peak current between 0.3 and 10 μA was discarded. Data derived from all electrophysiologic experiments are reported as the mean ± SD of five experiments using different oocytes.

**Diazepam Modulation of Directly Activated and Potentiated GABA<sub>A</sub> Receptor Currents**

For direct activation experiments, oocytes were perfused with 10 μM propofol with or without 200 μM flumazenil in ND-96 buffer for 5 minutes and then stepped into the same solution plus diazepam for 5 additional minutes. For agonist potentiation experiments, each oocytes were perfused with 2 μM GABA plus 2 μM propofol with or without 200 μM flumazenil in ND-96 buffer containing for 5 minutes and then stepped into the same solution plus diazepam for 5 additional minutes. The normalized directly-activated or potentiated current amplitude was defined as the current amplitude recorded 1 minute after diazepam addition divided by the current amplitude recorded immediately preceding diazepam addition. The high concentration of flumazenil (200 μM) approaches the solubility of the drug in buffer and was chosen to reliably prevent diazepam action at the classical high-affinity benzodiazepine site. For potentiation experiments, we chose a GABA concentration (2 μM) that evokes a current...
amplitude in the presence of a clinically-relevant propofol concentration (2 µM) that is 10 –
20% that produced by 1 mM GABA and thus allows us to readily quantify current amplitude
enhancement or inhibition in recorded electrophysiological traces.

In the absence of flumazenil, diazepam actions on directly-activated and potentiated
current amplitudes were analyzed with Prism v8 software for MacOS (GraphPad, La Jolla,
CA) using their bell-shaped concentration–response equations:

\[
\begin{align*}
\text{Span}_1 & = 100 - \text{Dip} \\
\text{Span}_2 & = \text{Plateau} - \text{Dip} \\
\text{Section}_1 & = \frac{\text{Span}_1}{1 + 10^{-(\text{LogEC}_{50} - [\text{Diazepam}] )}} \\
\text{Section}_2 & = \frac{\text{Span}_2}{1 + 10^{-( [\text{Diazepam}] - \text{LogIC}_{50} )}} \\
\text{Normalized Peak Current Response} & = \text{Dip} + \text{Section}_1 + \text{Section}_2
\end{align*}
\]

where Dip is the maximum normalized current amplitude, Plateau is the normalized current
amplitude at infinitely high diazepam concentrations, EC\text{50} is the diazepam concentration that
evokes a normalized current amplitude that is mid-way between 100% and Dip, and IC\text{50} is the
concentration of diazepam that reduces the normalized current amplitude to one-half Dip. For
experiments to define the effects of diazepam on directly-activated current amplitudes in the
absence of flumazenil, Plateau was constrained to zero (i.e. no directly-activated current when
propofol action is completely inhibited by diazepam).

In the presence of flumazenil, diazepam actions on directly-activated and potentiated
current amplitudes were analyzed with Prism v8 software for MacOS using a Hill equation with
the maximum constrained to 100% and the slope to -1. For experiments defining the effects
of diazepam on directly-activated current amplitudes in the presence of flumazenil, the minimum was constrained to zero (i.e. no directly-activated current when propofol action is completely inhibited by diazepam).

Impact of Diazepam on the Propofol Concentration–Response Relationship in GABA_A Receptors.

For Schild analysis experiments, oocytes were perfused for 5 minutes with an ND-96 buffer solution containing 2 µM GABA, propofol (0 – 300 µM) and diazepam (0 – 10 µM) in the presence of 200 µM flumazenil. The resulting peak current amplitudes were normalized to those evoked by 1 mM GABA in the same oocyte. The relationship between these normalized amplitudes and the concentration of propofol was analyzed with Prism v8 software for MacOS (GraphPad) using their three parameter Hill coefficient with the bottom constrained to 0 and their Gaddum/Schild EC_50 shift model, the latter defined by the following equations:

\[
EC_{50} = 10^{LogEC_{50}}
\]

\[
Antag = 1 + \left( \frac{[\text{Diazepam}]}{10^{-1\cdot PA2}} \right)^{\text{Schild slope}}
\]

\[
LogEC = \log(EC_{50} \times Antag)
\]

\[
\text{Normalized Peak Current Response} = \frac{\text{Maximum}}{1 + 10^{(LogEC - [\text{Etomidate}]) \times n}}
\]

where Maximum is the peak normalized response at infinitely high propofol concentrations, \( EC_{50} \) is the propofol concentration that evokes a normalized response that is one-half of the Maximum, \( pA2 \) is the negative logarithm of the diazepam concentration needed to shift the propofol concentration–response curve by a factor of 2, and \( n \) is the Hill slope.
Impact of Diazepam and Flumazenil on the Anesthetic Potency of Propofol in Zebrafish Larvae

Zebrafish (Danio rerio) larvae (6 days postfertilization) contained in 96-well plates containing solutions of test compounds (propofol, flumazenil, diazepam) were placed in a Zebrabox (Viewpoint Behavioral Systems, Canada), exposed to 0.2 s light stimuli, and their responsiveness to those stimuli recorded and analyzed using Zebralab v3.2 software (Viewpoint Behavioral Systems) as previously described (McGrath et al. 2020). Data are reported as the mean ± SD probability of response derived from five plates. Data were fit with Prism v8 software for MacOS using a Hill equation in the form:

\[ \text{Normalized Photomotor Response Probability} = \frac{\text{Maximum}}{1 + 10^{-(\log EC_{50} - \text{Anesthetic})}} \]

where Maximum represents the normalized probability of response in the absence of anesthetic and EC_{50} is the anesthetic concentration required to obtain a probability of response that is one-half of the Maximum.

Statistical Analysis

Statistical comparisons between maximum current responses (GABA_{A} receptor studies) or anesthetic EC_{50}s (zebrafish larvae studies) were made using the extra sum-of-squares F test. When multiple comparisons were made (zebrafish larvae studies), a Bonferroni-adjusted P value was used to assess statistical significance. Ninety-five percent confidence intervals derived from non-linear least square curve fitting are given in parentheses. Sample sizes were based on our previous experience. Prism v8 software for
MacOS (GraphPad) was used for fitting and statistical testing with statistical significance assumed for $P < 0.05$. 
Results

Diazepam Modulation of Directly Activated GABA<sub>A</sub> Receptor Currents

To define the actions of diazepam on α<sub>1</sub>β<sub>3</sub>γ<sub>2L</sub> GABA<sub>A</sub> receptor currents that were directly activated by propofol, we first activated receptors for 5 minutes with 10 µM propofol. Immediately following this activation period, diazepam was added at concentrations ranging from 1 nM to 100 µM and the diazepam-induced change in current amplitude was quantified. Representative traces from such experiments performed in the absence of flumazenil and in the presence of 200 µM flumazenil are shown in figures 2A and 2B, respectively.

In the absence of flumazenil, directly activated current amplitudes progressively increased before decreasing with diazepam concentration. This produced a diazepam concentration-response relationship that was bell-shaped (figures 2A and 2C). Maximal potentiation of currents activated by propofol occurred at a diazepam concentration of 300 nM with a current response that was 190 ± 15% of the baseline response recorded in the same oocyte immediately before adding diazepam. At the highest concentration studied (100 µM), the current response was reduced by diazepam to 48 ± 16% of the baseline response. A fit of the diazepam concentration-current response relationship to a bell-shaped equation yielded an EC<sub>50</sub> for the current potentiating action of 38 nM (95% CI: 20 – 72 nM), an IC<sub>50</sub> for the current inhibiting action of 21 µM (95% CI: 15 – 29 µM), and a Dip that was 200% of the baseline response (95% CI: 180 – 210%).

In the presence of flumazenil, propofol-activated current amplitudes only decreased with diazepam concentration as the current potentiating action produced by nanomolar concentrations of diazepam observed in the absence of flumazenil was no longer present (figures 2B and 2C). A fit of the diazepam concentration-current response relationship to a Hill equation yielded an IC<sub>50</sub> for this inhibiting action of 32 µM (95% CI: 26 – 39 µM).
Diazepam Modulation of Potentiated GABA\textsubscript{A} Receptor Currents

In the next series of experiments, we quantified the concentration-dependent actions of diazepam on $\alpha_1\beta_3\gamma_2L$ GABA\textsubscript{A} receptor-mediated currents that were activated by 2 µM GABA and potentiated by 2 µM propofol. In the absence of flumazenil and as we observed with propofol-activated current amplitudes, propofol-potentiated current amplitudes first increased with diazepam concentration before progressively decreasing to produce a bell-shaped diazepam concentration-response relationship (figures 3A and 3C). Maximal diazepam-induced potentiation of these currents occurred at a diazepam concentration of 1 µM with a current response that was 170 ± 26% of that recorded in the same oocyte in the absence of diazepam. At the highest concentration studied (100 µM), diazepam reduced the current response to 67 ± 11% of the baseline response. A fit of the diazepam concentration-response relationship to a Bell-shaped equation yielded an EC\textsubscript{50} for the potentiating action of 130 nM (95% CI: 38 – 440 nM), an IC\textsubscript{50} for the inhibiting action of 9.0 µM (95% CI: 3.0 – 27 µM), a Dip that was 180% (95% CI: 140 – 210%) of the baseline response, and a plateau value at high diazepam concentrations that was 50% (95% CI: 26 – 75%) of the baseline response.

In the presence of flumazenil (and as we observed with propofol-activated current amplitudes), agonist-potentiated current amplitudes only decreased with diazepam concentration (figures 3B and 3C). A fit of the diazepam concentration-response relationship to a Hill equation yielded an IC\textsubscript{50} for this inhibiting action of 3.2 µM (95% CI: 1.9 – 5.2 µM) and a plateau value at high diazepam concentrations that was 32% (95% CI: 24 – 40%) of the baseline response.

Competitive Interactions between Propofol and Diazepam
To test whether the inhibitory action of diazepam on propofol action was competitive in nature, we defined its impact on the propofol concentration-response curve for potentiating GABA-elicited GABAA receptor currents. We focused on potentiation rather than direct activation because the former occurs at propofol concentrations that are clinically relevant and below those associated with potentially confounding propofol-induced GABAA receptor inhibition (Pistis et al., 1997). The experiments were performed using a fixed GABA concentration of 2 µM plus 200 µM flumazenil. Figure 4 shows that in the absence of diazepam, propofol-induced agonist potentiation increased with propofol concentration before reaching a plateau. A fit of the propofol concentration-response relationship for the potentiation of GABA-elicited current to a Hill equation yielded an EC50 for the potentiating action of 6.0 µM (95% CI: 3.2 – 11 µM) and a plateau value at high propofol concentrations that was 65% (95% CI: 57 – 74%) of that elicited by 1 mM GABA. With increasing diazepam concentrations, the propofol concentration-response relationship shifted to the right without a statistically significantly reduction in the maximal response recorded at high propofol concentrations (P = 0.43; table 1). At the highest concentration studied (10 µM), diazepam increased the propofol EC50 by 5-fold to 31 µM (95% CI: 15 – 64 µM). Such behavior is consistent a competitive interaction between diazepam and propofol. We then did a Schild analysis of the datasets. This yielded a propofol EC50 of 4.6 µM (95% CI: 3.2 – 6.8 µM), a pA2 of 6.1 (95% CI: 5.4 – 6.7), a Schild slope of 0.7 (95% CI: 0.2 – 1), a Hill slope of 1.6 (95% CI: 1.1 – 2.1), and a KB of 0.9 µM (95% CI: 0.19 – 4.0 µM). We then refit the data with the slope constrained to 1 to obtain an estimated diazepam dissociation constant of 1.7 µM (95% CI: 1.0 – 2.8 µM).

Impact of Diazepam/Flumazenil on the Sedative-Hypnotic Activity of Propofol
We have previously reported that in the presence of flumazenil, micromolar concentrations of diazepam competitively inhibit etomidate binding to the \( \beta^+/\alpha^- \) subunit interfacial sites and rightward shift the etomidate concentration-response curve for positively modulating GABA\(_A\) receptor currents (McGrath et al., 2020). This inhibitory action was associated with a large rightward shift in the etomidate concentration-response curve for abolishing the light-stimulated activity of zebrafish, providing further evidence of a mechanistic link between (1) etomidate binding to the \( \beta^+/\alpha^- \) subunit interfacial GABA\(_A\) receptor sites with resultant positive allosteric modulation and (2) the production of hypnosis in the zebrafish model. As the present studies show that such concentrations of diazepam similarly inhibit the positive modulatory actions of propofol on this receptor, we tested whether it would also rightward shift the propofol concentration-response curve for light-stimulated activity of zebrafish. Such a finding would support these interfacial receptor sites as also being critical for the immobilizing actions of propofol.

Figure 5 shows that both propofol (panel A) and etomidate (panel B) produce concentration-dependent reductions in light-stimulated zebrafish larvae motor responses. In the absence of any other drugs, the propofol EC\(_{50}\) for reducing light-responsiveness was 0.75 \( \mu \text{M} \) (95% CI: 0.46 – 1.2 \( \mu \text{M} \)) whereas that for etomidate was 0.66 \( \mu \text{M} \) (95% CI: 0.42 – 1.0 \( \mu \text{M} \)). While the presence of 200 \( \mu \text{M} \) flumazenil alone had no statistically significant impact on either anesthetic EC\(_{50}\), the combination of 200 \( \mu \text{M} \) flumazenil and 50 \( \mu \text{M} \) diazepam produced a very large and highly statistically significant 18-fold increase in the etomidate EC\(_{50}\) to 12 \( \mu \text{M} \) (95% CI: 4.7 – 36 \( \mu \text{M} \)) while producing no statistically significant effect on the propofol EC\(_{50}\) (table 2).
Discussion

In this manuscript, we describe studies to define the impact of diazepam on the *in vitro* GABA<sub>A</sub> receptor positive allosteric modulatory and *in vivo* anesthetic actions of propofol. We found that diazepam has two concentration-dependent actions on propofol-activated and potentiated GABA<sub>A</sub> receptor currents. At nanomolar concentrations, diazepam increased the amplitudes of such currents by up to 2-fold. These positive modulatory actions were abolished by flumazenil, indicating its mediation via the high affinity benzodiazepine site located at the α<sup>+</sup>/γ<sup>−</sup> subunit interface in the extracellular domain. Conversely, at micromolar concentrations, diazepam reduced these amplitudes in a flumazenil-insensitive manner. Studies of potentiation showed that this inhibitory action was surmountable by high concentrations of propofol and produced a rightward shift in the propofol concentration-response curve characterized by a Schild slope that was not statistically significantly different from one. Considering previous studies demonstrating overlapping binding sites for diazepam and propofol at the β<sup>+</sup>/α<sup>−</sup> subunit interface in the transmembrane domain along with electrophysiological studies indicating that diazepam binds to these transmembrane sites at micromolar concentrations, we conclude that the inhibition of propofol action by diazepam reflects a competitive interaction between the two drugs at these transmembrane receptor sites (Walters et al., 2000; Drexler et al., 2010; Kim et al., 2020). However, despite having antagonistic actions on both propofol-activated and potentiated GABA<sub>A</sub> receptor currents, diazepam had no statistically significant effect on the anesthetic potency of propofol in a zebrafish larvae model even when present at a near aqueous saturating concentration.

The current results may be compared to those we obtained previously with analogous studies of etomidate. In those studies, diazepam similarly produced flumazenil-inhibitable potentiation of etomidate-activated currents at nanomolar concentrations and flumazenil-
insensitive competitive antagonism of etomidate action at micromolar concentrations (McGrath et al., 2020). However, the diazepam dissociation constant derived from the Schild analysis in that study of etomidate was an order of magnitude higher than that derived in the current studies of propofol (18 µM versus 1.7 µM, respectively). This may reflect subtle conformational differences between receptors activated by etomidate alone versus those activated by GABA and potentiated by propofol, an explanation supported by structural studies showing that different positive allosteric modulators induce different GABA<sub>A</sub> receptor conformational states (Masiulis et al., 2019; Kim et al., 2020). A second difference is that while the drug combination of 50 µM diazepam plus 200 µM flumazenil increased the anesthetic EC<sub>50</sub> of etomidate by 18-fold, it had no statistically significant effect on the anesthetic EC<sub>50</sub> of propofol. This difference suggests that propofol induces anesthesia via mechanisms that are distinct from those of etomidate, with the former involving additional target sites beyond the β<sup>+</sup>/α<sup>−</sup> subunit interfacial sites of the GABA<sub>A</sub> receptor. These include other GABA<sub>A</sub> receptor sites where diazepam does not bind and thus act as a competitive propofol antagonist along with non-GABAergic targets which are modulated by clinical concentrations of propofol.

Although our experiments were not designed to define the locations of these other propofol target sites, previous studies offer possibilities. Using a photoprotection assay, Chiara et al. showed that propofol not only inhibits photoaffinity labeling by a photoreactive etomidate analog (R-[<sup>3</sup>H]azietomidate) at the two GABA<sub>A</sub> receptor β<sup>+</sup>/α<sup>−</sup> subunit interfacial sites, it also inhibits labeling by a photoreactive barbiturate analog (R-[<sup>3</sup>H]mTFD-MPAB) to a homologous class of transmembrane sites located at the α<sup>+</sup>/β<sup>−</sup> and γ<sup>+</sup>/β<sup>−</sup> interfaces (Chiara et al., 2013). This was interpreted to mean that propofol binds to both classes of sites. Of note, the apparent affinity of propofol for these two classes of sites were quite similar (IC<sub>50</sub>: 32 ± 12
µM and 49 ± 5 µM, respectively). While this may have been a coincidence, it raises the possibility that by binding to the two β⁺/α⁻ sites, propofol not only competitively inhibits R-[³H]azietomidate labeling to these sites but also allosterically inhibits R-[³H]mTFD-MPAB labeling to the α⁺/β⁻ and γ⁺/β⁻ sites. Such crosstalk among inter-subunit transmembrane anesthetic binding sites has been documented in mutagenesis studies of GABA_A receptors and would explain why subsequent cryo-electron microscopy studies failed to detect propofol binding to the α⁺/β⁻ and γ⁺/β⁻ sites (Szabo et al., 2019). Separate photolabeling studies using the photoactivatable propofol analog ortho-propofol diazirine identified another potential propofol binding site within the β subunit at the interface between the transmembrane and extracellular domains (Yip et al., 2013). Surprisingly, there was no photolabeling by this analog of the β⁺/α⁻ sites and no studies to show that propofol itself could competitively inhibit ortho-propofol diazirine photolabeling of this intra-subunit site. Additionally, subsequent cysteine-modification protection studies revealed that propofol does not protect this site from p-chloromercuribenzenesulfate modification (Stern and Forman, 2016). Thus, it is uncertain whether the propofol photolabel used in these studies binds to the exact same GABA_A receptor site(s) as propofol.

In addition to GABA_A receptors, other protein targets may contribute to the anesthetic actions of propofol (Tang and Eckenhoff, 2018). In particular, glycine receptors have been suggested to play an important role in the anesthetic actions of propofol, but not etomidate (Jin et al., 2015). They are the major inhibitory neurotransmitter receptor in the brainstem and spinal cord, are structurally similar to and often colocalize with GABA_A receptors, and have been suggested to play a role in the actions of inhaled anesthetics (Harrison et al., 1993; Sonner et al., 2003; Zhang et al., 2003; Franks, 2006). Similar to GABA_A receptors, they are positively modulated by propofol, selective for chloride ions, and produce hyperpolarization...
resulting in central nervous system depression when activated. Voltage-gated ion channels and hyperpolarization-activated cyclic nucleotide-regulated (HCN) channels are also modulated by propofol, but their potential roles in mediating anesthesia are less clear (Ouyang et al., 2003; Chen et al., 2005; Chen et al., 2009).

The present studies have implications regarding the potential clinical development of anesthetic competitive antagonists capable of quickly and reliably reversing the actions of general anesthetic agents. They indicate that while it may be possible to reverse all of the anesthetic actions of agents that act highly selectively at the transmembrane anesthetic binding sites (e.g. etomidate) using the flumazenil/diazepam drug combination, such reversal is unlikely to be achieved for drugs which involve additional targets (e.g. propofol).

We acknowledge that there are limitations to the present studies. First, although the Schild analysis assumes that the competitive antagonist is a null modulator, electrophysiological studies have shown that diazepam binding to the transmembrane anesthetic binding sites on the GABA<sub>A</sub> receptor modestly impacts receptor function (Walters et al., 2000; McGrath et al., 2021). Specifically, diazepam enhances GABA<sub>A</sub> receptor function when bound to the β<sup>+</sup>/α<sup>−</sup> sites and reduces it when bound to the γ<sup>+</sup>/β<sup>−</sup> site. As these opposing actions tend to offset one another in wild-type receptors resulting in no change in GABA potency, we believe that their impact on our Schild analysis is likely small (McGrath et al., 2021). Second, we only studied one GABA<sub>A</sub> receptor subtype, albeit one that is thought to play a central role in the actions of general anesthetic agents. Other subtypes likely also contribute to these actions, including extra-synaptic ones that lack the γ subunit and are thus insensitive to the nanomolar potentiating actions of diazepam (Orser, 2006). Finally, we used a zebrafish larvae assay rather than a mammalian one to assess the ability of the diazepam/flumazenil drug combination to reverse the immobilizing actions of propofol. We
chose this aquatic animal model because it allowed us to efficiently quantify the anesthetic potency of propofol using many animals at defined steady-state drug concentrations that are minimally impacted by protein binding and relatable to those used in our GABA_A receptor studies. It also allowed us to directly compare the results of our current studies with propofol to our previously published ones using etomidate (McGrath et al., 2020). Zebrafish GABA_A receptor subunits and the receptors they form are respectively structurally and functionally similar to those found in mammals (Sadamitsu et al., 2021). The zebrafish larvae that we used were 6 days post fertilization to allow sufficient upregulation of the NKCC2 chloride transporter and render GABAergic action inhibitory (Reynolds et al., 2008; Zhang et al., 2010). Potency measurements obtained using zebrafish larvae utilizing anesthetic drugs representing multiple different chemical classes closely correlate with those defined using tadpoles, a long-used animal model for anesthetic action (Yang et al., 2018). Thus, we believe that this is a reasonable animal model to study anesthetic mechanisms. Nevertheless, it must be recognized that there may be differences among animal species and behavioral models regarding the mechanisms of anesthesia.

In conclusion, our studies show that the GABA_A receptor positive allosteric modulatory actions of propofol – like those of etomidate – are competitively antagonized by micromolar concentrations of diazepam. However, in the case of propofol, such antagonism is not associated with a reduction in the immobilizing potency of propofol. This suggests that while both propofol and etomidate can modulate GABA_A receptors by binding to the β+/α- subunit interfacial sites, propofol-induced anesthesia likely involves additional target sites.
Authorship Contributions

Participated in research design: Pence, Hoyte, McGrath, Forman, Raines

Conducted experiments: Pence, Hoyte

Performed data analysis: Pence, Hoyte, Raines

Wrote or contributed to manuscript writing: Pence, Hoyte, McGrath, Forman, Raines
References


Funding Footnote

No author has an actual or perceived conflict of interest with the contents of this article.

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Figure Legends

**Figure 1:** Cryo-electron microscopy image of the $\alpha_1\beta_2\gamma_2L$ GABA$_A$ receptor with diazepam bound. Cryo-electron microscopy studies (protein data bank: ID 6X3X) demonstrate diazepam binding to both the classical benzodiazepine binding site located within the GABA$_A$ receptor extracellular domain at the $\alpha^+\gamma$ subunit interface and the etomidate binding sites located within the GABA$_A$ receptor transmembrane domain at the two $\beta^+/\alpha^-$ subunit interfaces (Kim et al., 2020). The $\alpha$, $\beta$, and $\gamma$ subunits are light blue, dark blue, and teal colored, respectively. This figure was created using UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the university of California, San Francisco, with support from NIH P41-GM10331. (Pettersen et al., 2004)

**Figure 2.** Diazepam modulation of $\alpha_1\beta_3\gamma_2L$ GABA$_A$ receptor currents that were activated by 10 µM propofol in the absence or presence of flumazenil. (A) Representative traces showing the impact of diazepam at the indicated concentrations on propofol-activated currents in the absence of flumazenil. (B) Representative traces showing the impact of diazepam at the indicated concentrations on propofol-activated currents in the presence of 200 µM flumazenil. The dashed lines indicate the baseline response recorded immediately prior to diazepam addition. The gray boxes indicate the time when diazepam was present. The green and red arrows respectively highlight the diazepam-induced potentiation and inhibition of propofol-activated currents. (C) Diazepam concentration-response curves for 10 µM propofol-activated current amplitudes in the absence or presence of flumazenil (200 µM). Each data point represents the mean ± SD derived from five different oocytes. Each curve is a nonlinear least-squares fit of the dataset to either a bell-shaped equation (absence of flumazenil) or a
Hill equation (presence of flumazenil). In the absence of flumazenil, the diazepam EC50 and IC50 were 38 nM (95% CI: 20 – 72 nM) and 21 µM (95% CI: 15 – 29 µM), respectively. In the presence of flumazenil, the diazepam IC50 was 32 µM (95% CI: 26 – 39 µM). Insets show the chemical structures of diazepam and flumazenil.

Figure 3. Diazepam modulation of $\alpha_1\beta_3\gamma_2L$ GABA<sub>A</sub> receptor currents that were activated by 2 µM GABA and potentiated by 2 µM propofol in the absence or presence of flumazenil. (A) Representative traces showing the impact of diazepam at the indicated concentrations on currents activated by GABA and potentiated by propofol in the absence of flumazenil. (B) Representative traces showing the impact of diazepam at the indicated concentrations on currents activated by GABA and potentiated by propofol in the presence of 200 µM flumazenil. The dashed lines indicate the baseline response recorded immediately prior to diazepam addition. The gray boxes indicate the time when diazepam was present. (C) Diazepam concentration-response curves for GABA-activated and propofol-potentiated current amplitudes in the absence or presence of flumazenil. Each data point represents the mean ± SD derived from five different oocytes. Each curve is a nonlinear least-squares fit of the dataset to either a bell-shaped equation (absence of flumazenil) or a Hill equation (presence of flumazenil). In the absence of flumazenil, the diazepam EC50 and IC50 were 130 nM (95% CI: 38 – 440 nM) and 9.0 µM (95% CI: 3.0 – 27 µM), respectively. In the presence of 200 µM flumazenil, the diazepam IC50 was 3.2 µM (95% CI: 1.9 – 5.2 µM).

Figure 4. Modulation by diazepam of the propofol concentration-response relationship for the potentiation of 2 µM GABA elicited $\alpha_1\beta_3\gamma_2L$ GABA<sub>A</sub> receptor currents. Flumazenil (200 µM) was present in all experiments to prevent diazepam modulation from the classical benzodiazepine binding site. Each data point represents the mean ± SD derived from five
different oocytes. The curves are derived from a Schild analysis of the data and yielded a propofol EC$_{50}$ in the absence of diazepam of 4.6 µM (95% CI: 3.2 – 6.8 µM), a pA2 (in Molar) of 6.1 (95% CI: 5.4 – 6.7), a Schild slope of 0.7 (95% CI: 0.2 – 1), a Hill slope of 1.6 (95% CI: 1.1 – 2.1), and a K$_B$ of 0.9 µM (95% CI: 0.2 – 4 µM).

**Figure 5. Impact of 50 µM diazepam and 200 µM flumazenil on the propofol and etomidate concentration–response curves for abolishing zebrafish larvae photomotor responses to a light stimulus.** Each data point represents the mean ± SD normalized photomotor response from 40 larvae over five plates. The curves are nonlinear least squares fits of the datasets to a Hill equation. Insets show the chemical structures of propofol and etomidate. (A) Propofol concentration–response curves for abolishing zebrafish larvae motor responses to a light stimulus. Propofol alone abolished responsiveness in a concentration-dependent manner with an EC$_{50}$ of 0.75 µM (95% CI: 0.46 – 1.2 µM). Neither the presence of 200 µM flumazenil nor the combination of 200 µM flumazenil plus 50 µM diazepam had any significant effect on the propofol EC$_{50}$ (95% CI: 1.1 µM and 0.85 µM, respectively). (B) Etomidate concentration–response curves for abolishing zebrafish larvae motor responses to a light stimulus. Etomidate data is from previously published studies (McGrath et al., 2020). Etomidate alone abolished responsiveness with an EC$_{50}$ of 0.66 µM (95% CI: 0.42 – 1.0 µM). Flumazenil (200 µM) produced a relatively small (~2-fold) statistically insignificant increase in the etomidate EC$_{50}$ to 1.2 µM (95% CI: 0.86 – 1.8 µM) whereas the combination of 200 µM flumazenil plus 50 µM diazepam increased the etomidate EC$_{50}$ by 18-fold to 12 µM (95% CI: 4.7 – 31 µM).
Table 1: Impact of Diazepam on the Propofol Concentration-Response Relationship for Potentiation of GABA\textsubscript{A} Receptors*

<table>
<thead>
<tr>
<th>[Diazepam]</th>
<th>Maximum Amplitude#</th>
<th>Propofol EC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µM</td>
<td>65% (95% CI: 57 – 74%)</td>
<td>6.0 µM (95% CI: 3.2 – 11 µM)</td>
</tr>
<tr>
<td>2.5 µM</td>
<td>58% (95% CI: 48 – 69%)</td>
<td>16 µM (95% CI: 7.8 – 32 µM)</td>
</tr>
<tr>
<td>5.0 µM</td>
<td>64% (95% CI: 53 – 74%)</td>
<td>24 µM (95% CI: 13 – 44 µM)</td>
</tr>
<tr>
<td>7.5 µM</td>
<td>59% (95% CI: 53 – 65%)</td>
<td>26 µM (95% CI: 18 – 38 µM)</td>
</tr>
<tr>
<td>10 µM</td>
<td>54% (95% CI: 45 – 65%)</td>
<td>31 µM (95% CI: 16 – 59 µM)</td>
</tr>
</tbody>
</table>

* Derived from a Hill equation with the bottom and Hill slope constrained to 0 and 1, respectively. The concentrations of GABA and flumazenil were 2 µM and 200 µM, respectively.

\# Maximum current amplitude at high propofol concentrations as a percentage of that elicited by 1 mM GABA
Table 2: Immobilizing Potencies of Propofol and Etomidate in the Absence or Presence of Flumazenil +/- Diazepam*

<table>
<thead>
<tr>
<th>Drug(s)</th>
<th>Anesthetic Immobilizing EC$_{50}$</th>
<th>95% Confidence Interval</th>
<th>Bonferroni-Adjusted P Value Versus Anesthetic Alone#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propofol</td>
<td>0.75 µM</td>
<td>0.45 – 1.3 µM</td>
<td>NA</td>
</tr>
<tr>
<td>Propofol + Flumazenil</td>
<td>1.1 µM</td>
<td>0.44 – 2.9 µM</td>
<td>0.43</td>
</tr>
<tr>
<td>Propofol + Flumazenil + Diazepam</td>
<td>0.85 µM</td>
<td>0.51 – 1.4 µM</td>
<td>0.71</td>
</tr>
<tr>
<td>Etomidate</td>
<td>0.66 µM</td>
<td>0.42 – 1.0 µM</td>
<td>NA</td>
</tr>
<tr>
<td>Etomidate + Flumazenil</td>
<td>1.2 µM</td>
<td>0.86 – 1.8 µM</td>
<td>0.06</td>
</tr>
<tr>
<td>Etomidate + Flumazenil + Diazepam</td>
<td>12 µM</td>
<td>4.7 – 31 µM</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

*Derived from a Hill equation with the bottom and Hill slope constrained to 0 and -1, respectively. The [GABA] was 2 µM.

#Null hypothesis: EC$_{50}$ is the same as that measured for anesthetic alone.
Fig. 2

A

10 μM Propofol

1 nM Diazepam

100 μM Diazepam

2 min

30 nA

50 nA

60 nA

B

10 μM Propofol + 200 μM Flumazenil

1 nM Diazepam

1 μM Diazepam

100 μM Diazepam

2 min

150 nA

70 nA

50 nA

C

Normalized Directly-Activated Current Amplitude (% Baseline Response)

[Diazepam] μM

No flumazenil
+ 200 μM flumazenil

Diazepam

Flumazenil
Fig. 3

A

2 μM GABA + 2 μM Propofol

1 nM Diazepam

1 μM Diazepam

100 μM Diazepam

2 min

200 nA

500 nA

400 nA

B

2 μM GABA + 2 μM Propofol + 200 μM Flumazenil

1 nM Diazepam

1 μM Diazepam

100 μM Diazepam

2 min

300 nA

400 nA

300 nA

C

Normalized Agonist-Potentiated Current Amplitude (% Baseline Response)

Normalized Agonist-Potentiated Current Amplitude

0.001

0.1

1

10

1,000

[Diazepam] μM
Fig. 4
Fig. 5