A Novel Bifunctional Alkylphenol Anesthetic Allows Characterization of γ-Aminobutyric Acid, Type A (GABA$_A$), Receptor Subunit Binding Selectivity in Synaptosomes\textsuperscript{a,b}

Kellie A. Woll\textsuperscript{1,5}, Sruthi Murlidaran\textsuperscript{b}, Benika J. Pinch\textsuperscript{1}, Jérôme Hénin\textsuperscript{a**, b}, Xiaoshi Wang\textsuperscript{1, 5}, Reza Salari\textsuperscript{a**, b}, Manuel Covarrubias\textsuperscript{a**, b}, William P. Dailey\textsuperscript{1}, Grace Brannigan\textsuperscript{a**, b}, Benjamin A. Garcia\textsuperscript{a**, c}, and Roderic G. Eckenhoff\textsuperscript{1, 5, 7, 9}

From the Departments of\textsuperscript{4} Anesthesiology and Critical Care and \textsuperscript{5} Pharmacology and the \textsuperscript{6} Epigenetics Program, Department of Biochemistry and Biophysics, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania 19104, the \textsuperscript{4} Center for Computational and Integrative Biology and \textsuperscript{6} Department of Physics, Rutgers University, Camden, New Jersey 08102, the \textsuperscript{1} Department of Chemistry, University of Pennsylvania School of Arts and Sciences, Philadelphia, Pennsylvania 19104, the \textsuperscript{2} Laboratoire de Biochimie Théorique, Institut de Biologie Physico-Chimique, CNRS UMR 8251 and Université Paris Diderot, 5013 Paris, France, and the \textsuperscript{8} Department of Neuroscience and Farber Institute for Neuroscience, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Propofol, an intravenous anesthetic, is a positive modulator of the GABA$_A$ receptor, but the mechanistic details, including the relevant binding sites and alternative targets, remain disputed. Here we undertook an in-depth study of alkylphenol-based anesthetic binding to synaptic membranes. We designed, synthesized, and characterized a chemically active alkylphenol anesthetic, propofol (Fig. 1), has been strongly implicated as a modulator of the GABA$_A$ receptor. Relatively low concentrations of this alkylphenol significantly potentiate GABA-induced current, an action that hyperpolarizes the postsynaptic membrane and thereby likely contributes to hypnosis and possibly other anesthesia phenotypes (10, 11). Furthermore, multiple reports indicate that phasic inhibition is particularly sen-

γ-Aminobutyric acid (GABA) is well established as the major inhibitory neurotransmitter within the adult mammalian brain. The majority of GABA inhibitory activity is a consequence of binding to a set of pentameric ligand-gated ion channels called the GABA$_A$ receptor. GABA$_A$ receptors are largely heteromeric protein complexes composed of five different subunits that form a central pore that mediates chloride flux. Including the multiple isoforms, heterogeneity of the receptor is extensive with potentially more than 800 combinations (1). This complexity can be partially simplified by the degrees of selective cellular localization for some subunits and isoforms. Synaptic GABA$_A$ receptors contribute considerably to the communication between neurons, including influencing presynaptic neurotransmitter release as well as inducing postsynaptic hyperpolarization (1–4). Numerous studies have indicated that synaptic GABA$_A$ receptors are predominantly of a 2α:2β:1 γ stoichiometry (5, 6) that organizes in an alternating order (e.g. αγβαβ anti-clockwise as seen from synaptic cleft) (5–7). The resulting complex yields an abundance of potential ligand interaction surfaces within one heteropentamer, including at least four unique subunit interfaces. As such, it is justified that the composition and orientation of subunits are functionally significant, with different pharmacological properties pertaining to different GABA$_A$ receptor complexes (1, 8).

Numerous drugs influence GABA$_A$ receptor activity, including general anesthetics that are used extensively in modern medicine and in scientific research (9). For example, 2,6-dipropylphenol (propofol\textsuperscript{2} (Fig. 1) has been strongly implicated as a modulator of the GABA$_A$ receptor. Relative low concentrations of this alkylphenol significantly potentiate GABA-induced current, an action that hyperpolarizes the postsynaptic membrane and thereby likely contributes to hypnosis and possibly other anesthesia phenotypes (10, 11). Furthermore, multiple reports indicate that phasic inhibition is particularly sen-

\textsuperscript{a} This work was supported by National Institutes of Health Grants GM055876 and GM107174, Department of Defense Grant BC123187P1, and National Science Foundation Graduate Research Fellowship DGE-1321851. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

\textsuperscript{b} To whom correspondence should be addressed: 311A John Morgan Bldg., 3620 Hamilton Walk, Philadelphia, PA 19104-6122. Tel.: 215-746-8699; Fax: 215-349-5078; E-mail: roderic.eckenhoff@uphs.upenn.edu.

\textsuperscript{2} The abbreviations used are: propofol, 2,6-diisopropylphenol; ABPP, affinity-based protein profiling; MD, molecular dynamics; AFEP, alchemical free energy perturbation; 1-AMA, 1-aminoanthracene; MS, mass spectrometry; TMT, tandem mass tag; propofol, 2-fluoro-1,3-diisopropylbenzene; CI, confidence interval.

\textsuperscript{*} This article contains supplemental Table S1 and S2–S32.

\textsuperscript{†} The JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 291, NO. 39, pp. 20473–20486, September 23, 2016 © 2016 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
Sensitive to low concentrations of propofol, suggesting that synaptic GABAergic signaling is a critical pathway for the anesthetic’s pharmacological effects (12–14).

Investigations have focused on the potential binding sites within heterologously expressed αβγ GABA\textsubscript{A} receptors. A wide range of mutagenesis studies have probed ligand-gated ion channel electrophysiology and have shown that mutation of various residues predicted to reside within subunit interfacial regions alters propofol modulation (9, 15–17). Particular point mutations within β subunits, such as Asn-265, greatly decreased propofol-positive modulation (11, 18). Our previous work using the tritiated photoaffinity ligand (PAL) meta-azipropofol demonstrated frequent labeling of interfacial residues within the heterologously expressed Cys loop superfamily of receptors, including α,β,γ GABA\textsubscript{A} receptors (19). These findings further suggest that subunit interfaces are potentially involved in propofol modulation. Structure-activity relationships applying alkylphenol analogues and/or other chemical derivatives (20, 21), molecular dynamic (MD) simulations (22, 23), as well as other investigations have suggested complex physicochemical interactions between propofol and GABA\textsubscript{A} receptors (24). Together, these studies have provided insight regarding the potential mechanism by which propofol perturbs GABA\textsubscript{A} receptor protein dynamics. However, in addition to the biased nature of using heterologously expressed receptors, it is recognized that each method has experimental limitations that result in the current uncertainty regarding alkylphenol interactions within the receptor.

Our objective was to advance the current understanding of anesthetic interactions with heteromeric receptors by addressing the interaction(s) of alkylphenols with GABA\textsubscript{A} receptors within their native synaptic milieu. Our approach applied a novel chemically active alkylphenol anesthetic for quantitative affinity-based protein profiling (ABPP) of propofol within synaptosomes. By utilizing a native tissue-derived system, the relative GABA\textsubscript{A} receptor subunit expression, pentameric composition, protein-protein interactions, and lipid milieu are maintained. We assessed these binding results with independent MD simulations using the alchemical free energy perturbation (AFEP) algorithm (25) to predict potential molecular recognition elements within α,β,γ GABA\textsubscript{A} receptor-binding sites. Finally, we examined the impact of these molecular recognition elements within the synaptic GABA\textsubscript{A} receptors with photoaffinity protection experiments. Our studies led to the unbiased identification of GABA\textsubscript{A} receptor subunits in native synaptic membranes as alkylphenol-binding proteins. Our investigation suggested higher affinity interactions for β+/α− and α+/β− interfacial sites relative to γ-containing subunit interfaces and hydrogen bonding as the major recognition element for the alkylphenol-GABA\textsubscript{A} receptor complex.

**Results**

*Synthesis of AziPm-click (1) —*To identify the alkylphenol-binding proteins within the synaptic proteome, we developed 2-((prop-2-yn-1-yl)oxy)methyl)-5-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenol, or AziPm-click (1), a photoaffinity tandem bioorthogonal alkylphenol anesthetic ligand (Fig. 1). AziPm-click (1) was designed to integrate two chemically active groups that allow for ABPP as follows: 1) a diazirine photoreactive group to covalently label protein interaction sites, and 2) an alkynyl group for covalent attachment of a reporter tag by 1,3-dipolycycloaddition reaction (e.g. “Click Chemistry”) to capture and identify photoaffinity labeled proteins within the synaptic proteome.

*Synthesis of AziPm-click (1), shown in Scheme 1 (described in supplemental S2–S7), starts with the previously reported 4-bromo-2-(methoxymethoxy)-1-methylbenzene (2) (26). Conversion of 2 to the Grignard reagent using magnesium in THF followed by treatment with pyridinium dichromate produced trifluoromethyl ketone 3. Conversion of 3 to the oxime 4 and oxime tosylate 5 followed standard procedures. Treatment of 5 with excess liquid ammonia produced diaziridine 6 that was oxidized to the diazirine 7 using pyridinium dichromate. Benzyl bromination using N-bromosuccinimide produced 8, which was treated with the sodium salt of propargylic alcohol in tetrahydrofuran to provide 9. Removal of the methoxymethyl protecting group in the presence of the propargylic ether required carefully controlled conditions and was finally accomplished using sodium hydrogen sulfate-impregnated silica gel in methylene chloride (27).
Physicochemical and Protein-binding Properties—The physicochemical characteristics of propofol and AziPm-click (1) are summarized in Table 1, and the geometry-optimized structure is shown in Fig. 2A. The UV absorption spectrum of AziPm-click (1) shows a well-defined peak between 330 and 400 nm due to the diazirine group (methanol extinction coefficient $\Sigma_{365\text{ nm}}$ of 580 M$^{-1}$ cm$^{-1}$). Over the course of UV irradiation using a Rayonet RPR-3500 lamp within the aqueous solution, the AziPm-click (1) diazirine absorbance band decreased intensity indicating photoactivation (Fig. 2B). The time-dependent photoreactivity of AziPm-click (1) in aqueous solution was a single exponential decay with a half-life ($t_{1/2}$) of 25 min (95% CI; 20–33) within a 1-cm path length cuvette and 6 cm from the lamp. To confirm retention of other major molecular recognition features, we compared equilibrium binding affinities of applied alkylphenol general anesthetics with the model protein apoferritin by isothermal calorimetry and 1-aminoanthracene (1-AMA) competition (28, 29). The affinities of alkylphenols for apoferritin have shown to be well correlated with GABA$_A$ receptor potentiation (30–32); results are summarized in Table 2.

Fluorescent Profiling of Alkylphenol-binding Proteins—To confirm the functionality of both the chemically active groups for downstream ABPP, we employed AziPm-click (1) within mouse synaptosomes using an azide-PEG3-Alexa 488 fluorophore as a reporter tag. The fluorescent labeling of proteins was reliant on UV exposure (Fig. 3A). Fluorescent labeling was decreased with increased concentrations of propofol indicating protection of alkylphenol-binding proteins within synaptosomes (Fig. 3B). To control for potential “inner filter” of UV light, ketamine was employed as a protecting ligand (Fig. 3C), which conferred no changes in fluorescence intensity seen in Fig. 3D.

$\alpha_1\beta_2\gamma_2L$ GABA$_A$ Receptor Electrophysiology with AziPm-click (1)—AziPm-click (1) was functionally active on $\alpha_1\beta_2\gamma_2L$ GABA$_A$ receptors heterologously expressed in Xenopus oocytes. AziPm-click (1) demonstrated similar positive modulation activity as propofol (Fig. 4, A and B). The EC$_{50}$ value for propofol-positive modulation (at a GABA EC$_{10}$) in our system was 10 $\mu$M (95% CI; 3.3–17). AziPm-click (1) required a higher concentration for a similar response with an EC$_{50}$ of 49 $\mu$M (95% CI; 38–61).

In Vivo Anesthetic Activity and Photoaffinity Labeling—Propofol and AziPm-click (1) demonstrated similar pharmacological end points within Xenopus laevis tadpoles, inducing reversible hypnosis with no observable toxicity summarized in Table 3 and shown in Fig. 4D. To indicate photoaffinity labeling of pharmacologically relevant targets, we demonstrated that AziPm-click (1) produced sustained anesthetic end points (immobility) in vivo after UV irradiation (33). X. laevis tadpoles were exposed to 12 $\mu$M AziPm-click (1) or 3 $\mu$M propofol for 30 min. Tadpoles were then exposed to 10 min of low intensity UV irradiation or were maintained as a 10-min non-UV irradiation control. Similar to our previous reports for meta-azipropofol (33), only tadpoles exposed to AziPm-click (1) and 10-min UV irradiation displayed prolonged immobility after drug washout (Fig. 4, D and E).

ABPP for Alkylphenol Anesthetics—The ABPP workflow using AziPm-click (1) with relative quantification is summarized in Fig. 5A. Azide-PEG3-biotin was employed as the reporter tag for streptavidin-affinity isolation of photoaffinity labeled protein targets. Tandem mass tag (TMT) isotopic label-
Propofol-binding Sites in Native Synaptic GABA\textsubscript{\textalpha} Receptor

![Image](image_url)

FIGURE 4. \(\alpha_{\textbeta_2}\gamma_2L\) GABA\textsubscript{\textalpha} receptor and anesthetic activity of AziPm-click (1). A, representative traces of ligand activity on heterologously expressed \(\alpha_{\textbeta_2}\gamma_2L\) GABA\textsubscript{\textalpha} receptors in \(X.\ laevis\) oocytes. Traces are shown with the oocyte responses to GABA EC\textsubscript{10} value and corresponding modulation propofol (3 \(\mu\)M) or AziPm-click (1) (20 \(\mu\)M). B, concentration-response curves for propofol (black circle) and AziPm-click (1) (green diamond) for the positive modulation of heterologously expressed GABA\textsubscript{\textalpha} receptor \(\alpha_{\textbeta_2}\gamma_2L\) in \(X.\ laevis\) oocytes. Each point represents the mean of four oocytes (\(n = 4\)) ± S.E., and data were fitted to a sigmoidal dose-response curve with variable Hill slope. C, dose-response curves for propofol (\(n = 210\); black circle) and AziPm-click (1) (\(n = 300\); green diamond) for loss of spontaneous movement in tadpoles. Data were fitted to a sigmoidal dose-response curve with variable Hill slope, and the EC\textsubscript{50} and Hill slope values are represented in Table 3. D, time course of recovery control for \(X.\ laevis\) tadpoles following propofol (\(n = 30\); black open circle) or AziPm-click (1) (\(n = 30\); green open diamond) equilibration and 10 min no UV treatment. E, time course of recovery for tadpoles following propofol (\(n = 30\); black filled circle) or AziPm-click (1) (\(n = 30\); green filled diamond) equilibration and 10 min of low intensity UV irradiation.

| TABLE 3 | Tadpole studies |
|----------|-----------------|
|          | EC\textsubscript{50} (95% CI; \(\mu\)M) | Hill slope (mean ± S.E.) |
| AziPm-click (1) | 6.1 (5.1–7.4) | 3.0 ± 0.54 |
| Propofol | 0.90 (0.84–0.97) | 3.4 ± 0.31 |

MD Simulations of Dynamic Propofol Interactions with \(\alpha_{\textbeta_2}\gamma_2L\) GABA\textsubscript{\textalpha} Receptor—To understand the apparent subunit specificity noted in the above experiments, MD simulations for alkylphenol anesthetic binding were generated with an \(\alpha_1\beta_3\gamma_2\) GABA\textsubscript{\textalpha} model derived from an \(\alpha_1\beta_1\gamma_2\) GABA\textsubscript{\textalpha} model used previously (35). Docking calculations to the entire pentamer identified at least one propofol pose in each subunit interface, \(\beta_1/\alpha_1\) (two sites), \(\alpha_1/\beta_1\), \(\alpha_1/\gamma_1\), and \(\gamma_1/\beta_1\), as shown in Fig. 6A. Other than the channel lumen, no alternate sites were consistently detected over multiple docking runs. Docking of AziPm-click (1) to the same model yielded overlapping sites, demonstrating that AziPm-click (1) is not sterically hindered from binding to the intersubunit sites, despite the larger molecular size. Furthermore, as shown in Fig. 6, AziPm-click docking simulations yield similar orientations to propofol suggesting common favorable interactions.

A receptor-propofol complex was constructed with one propofol molecule in the highest scoring pose for each subunit interface (Fig. 7A). The complex was embedded in a fully hydrated phosphatidylcholine membrane and simulated for 270 ns using traditional equilibrium MD with atomic resolution. In addition to allowing the propofol in the intersubunit space to equilibrate before the affinity calculations, we used this simulation to characterize and compare the microscopic interactions between propofol and the binding pocket across subunits.

At the conclusion of the traditional MD simulation, standard binding affinities for propofol in each of the four distinct sites were calculated using separate 24-ns AFEP simulations. The
AFEP method also involves running MD simulations but is designed to facilitate simultaneous calculation of average quantities appearing in the Zwanzig equation (36), an exact expression for the free energy difference between two states (e.g. bound and unbound) that inherently accounts for all entropic and enthalpic contributions. The results from the AFEP simulations indicate three higher affinity sites at the H9251/H11001/H9252/H11002 and two H9252/H11001/H9251/H11002 interfaces, with KD values similar to propofol EC50.

The particularly low affinity of propofol for the H11001/H9253/H9252/H11002 interface, which has one more polar residue than the other interfacial cavities (Fig. 8A), seemed potentially contradictory to an essential role for hydrogen bonding. As shown in Fig. 7B, however, the pKd values for different subunit interfaces were found to be strongly correlated (r2 = 0.94) with the probability (Phb) that the propofol hydroxyl would form at least one hydrogen bond with one of the cavity-lining residues. Propofol in either of the two sites with low KD values (α+/β− and β+/α−) had at least Phb > 0.8; for the two low affinity sites, this probability was significantly reduced (P< 0.3). Thus, although propofol affinity is correlated with propofol hydrogen bonding, propofol is less likely to form hydrogen bonds with the more hydrophilic +γ/β− interfacial cavity. This result was due to stable hydration of the +γ/β− cavity, due to interactions of water molecules with γSer-301 and γThr-
Propofol-binding Sites in Native Synaptic GABA<sub>A</sub> Receptor

281 (Fig. 7E). The water molecules compete for hydrogen bonding partners and interact unfavorably with the propofol isopropyl groups.

Within the highest affinity site at the α+/β− interface, propofol orients as a hydrogen donor to the carbonyl backbone of Leu-223 within the βM1 transmembrane helix (Fig. 7C) where a bulge in backbone hydrogen bonding is observed in crystal structures for both GluCl (37) and the GABA<sub>A</sub> receptor β<sub>1</sub> homopentamer (38). Similar behavior was observed in simulations of triiodothyronine bound to interfacial sites (39). In the β+/α− interface, propofol alternates rapidly between serving as a hydrogen acceptor for βM2:Thr-262 and donor for βM2:Asn-265 (Fig. 7D). The associated slight reduction in <i>PK<sub>D</sub></i> is consistent with the slight reduction in <i>P<sub>hb</sub></i> and the line of best fit.

The AFEP calculations yield an intermediate affinity of propofol for the γ−/α+ interfacial site. Residues of the γ-face, however, are nearly identical to those of the β-face, as shown in Fig. 8, B and C, and sequence differences among site residues are unable to account for the moderate differences in hydrogen bonding and affinity between γ−/α+ and the higher affinity β−/α+/β− site. Because hydrogen bonding of propofol to the M1 backbone is frequently observed for β− but not γ−, it is possible that sensitivity of fluctuations in M1 secondary structure to non-cavity residues causes the observed weak sequence dependence. If so, the result suggests a further uncertainty in interpretations of mutagenesis experiments and the underlying assumption that identified residues are contact residues.

**Propofol and 2-Fluoro-1,3-diisopropylbenzene (Fropofol) Protection of GABA<sub>A</sub> Receptor Subunits—** To experimentally evaluate the role of the alkylphenol hydroxyl in selective binding to sites within synaptic GABA<sub>A</sub> receptor subunits, we applied the fluorine-substituted analogue propofol (Fig. 9A) within protection experiments (40). Previously, propofol did not modulate or disrupt propofol potentiation of the GABA<sub>A</sub> receptor, and it did not cause immobilization at even 100-fold higher concentrations than propofol. In contrast, propofol did display similar binding as propofol to protein sites that were not dependent on
hydrogen bond interactions (40). Azide-PEG3-biotin was employed as the reporter tag for streptavidin-affinity isolation of protein targets photoaffinity labeled by AzidPm-click (1) with or without protection ligands propofol or fropofol. Protein levels of GABA_A receptor subunits were determined by Western blotting before (or “input”) and after (or “elute”) streptavidin capture of biotinylated proteins. All GABA_A receptor subunits were detected within synaptosomes prior to capture (Fig. 9A), consistent with synaptic localization of these subunits. After capture, only α and β subunits were detected. All GABA_A receptor α and β subunits showed significant decreases in capture efficiency when propofol was present during UV irradiation. Unlike propofol, fropofol was unable to protect GABA_A receptor α and β subunits from capture (Fig. 9, A and B).

**Discussion**

In this study we investigated the molecular mechanisms and placement of alkylphenol anesthetic binding to synaptic GABA_A receptors. This multifaceted study integrated an assessment of total synaptic GABA_A receptor binding relative to the native proteome with a targeted investigation of key molecular recognition elements that contribute to binding affinity. Our approach deployed a novel anesthetic PAL containing a click chemistry moiety for downstream quantitative ABPP, as well as AFEP MD simulations of receptor binding and photoaffinity protection experiments. Together, these studies add to the understanding of propofol pharmacology and the dynamic behaviors of heteropentameric receptors.

Previous work has shown that the ligand-gated receptors, GABA_A receptors in particular, are modulated by propofol. Site-directed mutagenesis within heterologous expression systems and animal models provides the advantage of demonstrating drug activity and pharmacological changes as well as candidate regions of drug binding. The recognized pitfalls of mutagenesis, like the inherent ambiguity of defined interaction regions, perturbation of native protein dynamics and/or genetic compensation, mandates that additional strategies be deployed to complement these investigations. Photoaffinity labeling has been one method used to further the understanding of anesthetic-binding sites (41). Multiple PALs for propofol have been reported (42, 43), including one from our own labo-

**FIGURE 8.** Sequence variation in interfacial binding sites of an α₁β₂γ₂ GABA_A receptor heteropentamer. A, sequence alignment of + and − subunit interfaces that contribute to the formation of the binding cavity. Bold and * residues denote key sequence variations in the interfacial binding sites. B and C, helices of the four distinct subunit interface pairs with α + β – interface as the reference pair. In all panels, side chains are colored by residue type as follows: polar (green), hydrophobic (white), acidic (red), and basic (blue). B, extended view and binding site cavity view of the α + β – interface reference pair with all cavity contributing side chain residues represented. C, helices of the four distinct subunit interface pairs are colored according to sequence differences with the α + β – interface as the reference subunit pair displaying identical (light blue), similar (white), and change in residue type (orange). Note that for a given interface, coloring of the + and − subunit backbone reflects sequence differences from α, and β, respectively. Cavity residues are labeled according to a prime-numbering system in which M2:16 is equivalent to Ile-271, Thr-266, and Thr-281 for α subunits from capture (Fig. 9, A and B).

**FIGURE 9.** Ligand protection of synaptic GABA_A receptor capture. A, chemical structure of fropofol, B, representative Western blots for GABA_A receptor subunits of input (lanes 2–5) and the corresponding elution (lanes 7–10) for synaptosomal samples exposed to AzidPm-click (1) (10 μM) with or without UV irradiation and with or without co-exposure with propofol (100 μM) or fropofol (100 μM). Lanes 1, 6, and 11 contain protein ladders. B, comparison of non-UV and UV capture with or without propofol or fropofol protection for each GABA_A receptor subunit; values are represented as the mean of four experiments ± S.E. of the fraction of the corresponding input sample. Data were analyzed by two-way analysis of variance with Tukey’s multiple comparison test comparing fraction captured between protection conditions for each subunit. Significant differences compared with UV-irradiated eluate protection without protection ligand are shown (***, p < 0.001; **, p < 0.01; *, p < 0.05).
Propofol-binding Sites in Native Synaptic GAB_{A} Receptor

Hydrogen Bonding Mediates Alkylphenol-based Anesthetic Binding to the GAB_{A} Receptor—Numerous interactions can contribute to propofol affinity within a protein cavity. These include multiple weak interactions, such as the hydrophobic effect, as well as the potential for stronger interactions, such as hydrogen bonding with the alkylphenol hydroxyl. From the \( \alpha_{1}\beta_{2}\gamma_{2} \) GAB_{A} receptor MD simulations, the higher relative affinities for the \( \beta^{+}/\alpha^{-} \) and \( \alpha^{+}/\beta^{-} \) interfaces corresponded with increased probability for hydrogen bond interaction(s) for \( \beta^{+}/M2 \) Thr-262 and Asn-265 or \( \beta^{-}/M1 \) Leu-223 at a conserved bulge in the M1 backbone. In contrast, the lowest affinity (\( \gamma^{+}/\beta^{-} \)) interface contains a larger number of potential hydrogen bonding side chains; however, this increases cavity hydration and causes displacement of propofol. Propofol bound to the \( \alpha^{+}/\gamma^{-} \) sites yielded an intermediate affinity and likelihood of hydrogen bonding. Although the \( \alpha^{+}/\gamma^{-} \) side chains are equivalent to those of the higher affinity \( \alpha^{+}/\beta^{-} \) site, we observed that the backbone carbonyl of \( \beta^{-}/M1 \) Leu-223 was significantly more likely to serve as a hydrogen bond acceptor than in the homologous residue, \( \gamma^{-}M1 \) Ile-238. The origin of this difference is likely subtle and dependent on any residues that affect M1 secondary structure, not just cavity residues.

To confirm the contribution of the hydroxyl to the alkylphenol-based anesthetic-binding site within captured synaptic GAB_{A} receptor subunits and the functional relevance of these sites, we introduced the fluorine-substituted and therefore hydrogen bond-null ligand propofol into our investigation. With the fluorine replacement of the alkylphenol hydroxyl, propofol displayed no activity on heterologously expressed \( \alpha_{1}\beta_{2}\gamma_{2} \) GAB_{A} receptors (40). Propofol does, however, bind to propofol-binding sites that are characterized primarily by the hydrophobic effect (40). We observed that propofol, unlike propofol, did not protect the \( \alpha \) or \( \beta \) subunits from AziP-{\textit{click}} (1) capture. These results confirm MD simulations that identified hydrogen bonding as a key element contributing to alkylphenol-based anesthetic binding to synaptic GAB_{A} receptors and that such interfacial sites are likely to contribute to the altered functional activity.

\textbf{Study Limitations—}Although the goal of this investigation was to reconcile propofol pharmacology with a complex binding proteome, some limitations are implicit in our studies, including points for future improvement. First, our analogue AziP-{\textit{click}} (1) showed a reduced potency for modulating \( \alpha_{1}\beta_{2}\gamma_{2} \) GAB_{A} receptors and a parallel decrease in anesthetic potency as compared with the parent propofol. However, this is well within what has been reported for the alkylphenol chemo-type (53), and it is probably a result of the additional bulk and electrostatically active diazirine and ether/alkyne groups, which reduce hydrophobicity. Furthermore, associated docking simulations demonstrated overlapping poses for propofol and AziP-{\textit{click}} (1). Finally, MD simulations using the AFEP algorithm use the parent compound propofol rather than AziP-{\textit{click}} (1), but they yield affinities that are consistent with results from AziP-{\textit{click}} (1).

Second, our current investigation is limited to the employed biological system, a synapticosomal fraction derived from whole brain, and therefore, it is focused on synaptic proteins, including synaptic GAB_{A} receptors. Propofol has previously been
shown to influence tonic receptor-mediated inhibition that is anticipated to contribute to observed hypnotic sedation and other anesthesia phenotypes, specifically amnesia (13, 14, 54). Because of the lack of extrasynaptic GABA_4 receptor subunits within our proteome (data not shown), we could not determine the binding character for these receptors.

Finally, although the presented quantitative ABPP strategy allows the first platform for identifying alkylphenol protein targets relative to a native biological milieu, it does not directly identify the photoaffinity labeled residues. In part, this is a result of the challenging elution/digestion of labeled proteins and large modification size (>600 Da) of the biotin PEG3-conjugated AziPm-click (1) modification. Currently available cleavable biotin-X-azole linkers have variable cleavage efficiency and/or require reagents that perturb downstream quantitative labeling for native tissue-derived systems (55). Our efforts for non-quantifiable capture using cleaved biotin linker and AziPm-click (1) identified modifications on higher abundance proteins like the voltage-dependent cation channel (data not shown) that corresponded with earlier reports (56); however, residue-level modifications on lower abundance proteins, like the GABA_4 receptor, remained undetected. Thus, we cannot confirm an interfacial location of sites in this study, although this location has been demonstrated in heterologous receptors (19). Future development of chemically active alkylphenol anesthetics, cleavable biotin linkers, as well as enhanced peptide enrichment and release methods may allow for increased capture efficiency permitting detection of modifications within the very low abundance photoaffinity labeled peptides.

**Additional Alkylphenol-based Anesthetic Syaptic Targets—** It is unlikely that a given drug will only bind and act on a single protein target within a proteome. In particular, the small general anesthetic molecules have been shown to bind to many different proteins (57). Although propofol is thought to have higher affinity for specific protein targets relative to volatile anesthetics, the projected affinities for major targets, as we observed with the GABA_4 receptor, still remain in the low micromolar range. Therefore, it is not surprising that a number of targets (~200) were captured due to the promiscuous binding associated with the general anesthetic. Whether the activity of every identified protein is altered upon alkylphenol binding is not clear and is not likely. However, some captured targets, in addition to the GABA_4 receptor, have been reported as being influenced by propofol. Examples include syntaxin-1A (58), N-methyl-D-aspartate receptor (59), potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1 (60), as well as voltage-gated calcium channels (61) and potassium channels (62), all of which may contribute to desirable and/or undesirable pharmacological effects.

**Concluding Remarks—** Although the GABA_4 receptor is considered to be an important target for general anesthetics, the mechanism of GABA_4 receptor modulation remains unclear. In this investigation, we aimed to further understand alkylphenol binding to native receptors and to evaluate the molecular recognition elements that mediate affinity. Our results indicate that propofol binds to the assembled receptor in an asymmetric pattern, with greater affinity for β+/α− and α+/β− interfaces. Hydrogen bonding and cavity hydration were found to be the likely defining factors that contribute to the differential interfacial affinity and functional activity. In addition, this work suggests that the alkylphenol anesthetic proteome is large and complex, providing the opportunity to modulate activity at many targets. Finally, this work adds to current methodologies used for the identification of anesthetic targets and a better understanding of allosteric binding interactions.

**Experimental Procedures**

**General Synthetic Procedures—** Reagents and solvents were all used as acquired from commercial sources. 1H, 13C, and 19F NMR spectra were obtained on either a Bruker DMX 500 MHz or a Bruker DMX 360 MHz nuclear magnetic resonance spectrometer. The detailed synthetic methodology and associated NMR spectra for intermediates and AziPm-click (1) are provided in the supplemental material. Purity of AziPm-click (1) was determined using reverse phase-HPLC with a C-18 analytical column with a 60-min gradient from 40 to 70% acetonitrile in 0.1% formic acid at a 1 ml/min flow at ambient temperature (21–22 °C). AziPm-click (1) was monitored for UV-Vis absorbance at 210 and 365 nm. The retention time for AziPm-click (1) was observed at 22.3 min with a purity of >96%.

**Physicochemical Properties—** The UV spectrum and extinction coefficient of the AziPm-click (1) diazirine absorption were obtained from known concentrations in methanolic solutions and gathered from the Varian Cary 300 Bio UV-visible spectrophotometer. Photoactivation of the diazirine was measured by the disappearance of the diazirine UV absorption peaks when exposed to 350 nm light (Rayonet RPR-3500 lamp) –6 cm from the light source. Maximum water solubility was approximated using the extinction coefficient. Calculated octanol/water partition coefficients were generated using XLOGP3 software 22 with default settings. The geometry-optimized structures for AziPm-click (1) was calculated at the B3LYP/6–311+G (2d,p) level of theory using Gaussian 09 (63).

**Isothermal Titration Calorimetry—** Isothermal titration calorimetry isotherms for binding to soluble protein model horse spleen apoferritin were conducted as reported previously (31) and were resolved using a VP-isothermal titration calorimetry microcalorimeter (MicroCal, Inc., Northampton, MA). Origin 5.0 software was used to best-fit thermodynamic parameters to the heat profiles.

**1-AMA Displacement Fluorescence Assay—** 1-AMA fluorescence inhibition has been reported as a reliable measurement of anesthetic occupation of the horse spleen apoferritin anesthetic site (64). 1-AMA displacement studies were conducted as described previously (29). The fluorescence intensity versus concentration data were fitted to variable slope Hill models to obtain the IC50 and Hill slope. The Ks value was calculated using the Cheng-Prusoff equation to correct for the presence of the 1-AMA competitors.

**Crude Synaptosome Preparation—** Mouse crude synaptosomes were prepared as reported previously (65) with modifications. Male C57/B6 mice (8–12 weeks) were deeply anesthetized with isoflurene and intracardially perfused with ice-cold phosphate-buffered saline (PBS; pH 7.4) before decapitation. Brains were extracted and homogenized in ice-cold isolation buffer (IB; 0.32 mM sucrose, 2.5 mM HEPES, 1 mM EDTA (pH 7.4))
Propofol-binding Sites in Native Synaptic GABA<sub>A</sub> Receptor

(10% w/v%) in the presence of protease and phosphatase inhibitors. The homogenate was centrifuged at 1,000 × g for 10 min at 4 °C. The resulting supernatant was decanted, and the pellet was homogenized with an equal volume of IB and centrifuged at 1,000 × g for 10 min at 4 °C. Both supernatants were pooled and were centrifuged at 1,000 × g for 10 min at 4 °C. The supernatant was decanted and centrifuged at 12,000 × g for 20 min at 4 °C. The pellet was washed twice by resuspension of the pellet in 2× volumes of IB and centrifuged at 12,000 × g for 15 min at 4 °C. The resulting crude preparation of synaptosomes, now entirely free of the euthanizing isoflurane, was used in following experiments. All following protein contents are measured using BCA assay (Thermo Scientific). Animal care and experimental procedures involving mice were carried out according to a protocol approved by the IACUC of the University of Pennsylvania.

Synaptosomal Photoaffinity Labeling—Synaptosomes were resuspended to 1 mg of protein/ml in HEPES buffer medium (in mM: NaCl: 5, KCl: 5, NaHCO<sub>3</sub>: 1.2, NaH<sub>2</sub>PO<sub>4</sub>: 1, MgCl<sub>2</sub>: 10, glucose, and 10 HEPES (pH 7.4)). Concentrations of Azipm-click (1) with or without the presence of concentrations of competitive ligands (propofol, ketamine, or fropofol) in dimethyl sulfoxide (DMSO) vehicle (<0.3% v/v) were added, and synaptosomes were gently vortexed for 10 s. The samples were allowed to equilibrate for 5 min before being transferred to a parafilm-sealed 1-mm path length quartz cuvette. The sample was then irradiated for 20 min at a peak bandwidth of 350 nm from the light source. Non-irradiated samples were left in the dark at ambient temperature (22–25 °C) for 1 h with mild agitation. The samples were left in the dark at ambient temperature (22–25 °C) for 1 h with mild agitation. Directly to each sample 4× volume chilled methanol, 1.5× chilled chloroform, and 3× chilled double distilled H<sub>2</sub>O were added. Samples were vortexed vigorously and centrifuged at 1,400 × g for 30 min at 4 °C. Both liquid layers were carefully removed, and the protein pellet was washed with 2 ml of 1:1 (v/v) chilled methanol/chloroform. Samples were centrifuged at 3,500 × g for 30 min at 4 °C. Protein pellets were briefly air-dried before further processing.

Fluorophore Conjugation for Proteome Detection—To 150 μg of photolabeled or control synaptosomes, 8 μl of 10% SDS in water and 2 μl of 1 μl dithiothreitol (DTT) in water were added. Samples were vortexed and heated at 65 °C for 10 min. After a brief cooling, final concentrations of 30 μM azide-PEG3-fluor 488 (Click Chemistry Tools), 2 μM tris(3-hydroxypropyltriazolylmethyl)amine (Sigma), 1 mM ascorbic acid (Sigma), and 1 mM CuSO<sub>4</sub>·5H<sub>2</sub>O (Sigma) were added to each sample and vortexed vigorously. The samples were left in the dark at ambient temperature (22–25 °C) for 20 min. All remaining procedures were conducted with restricted light exposure.

Sample Processing for ABPP Mass Spectrometry Studies—750 μg of biotin-conjugated protein sample was resuspended in 500 μl of 25 mM NH<sub>4</sub>HCO<sub>3</sub> and 6 mM urea in water. Next, 150 μl of 5% Triton X-100 in water, 50 μl of 10% SDS in water, and 1.5 μl of 0.5 M DTT were added. The samples were heated for 15 min at 65 °C. After briefly cooling, 14 μl of 0.5 M iodoacetamide in water was added, and the sample was left in the dark for 45 min. Insoluble debris was separated by centrifugation for 10 min at 14,000 × g. The supernatant was diluted to 4 ml with PBS, and 2 ml of PBS containing 100 μl of 50% streptavidin-agarose resin (Thermo Scientific) was added. Biotinylated proteins within the sample were captured over resin overnight at 4 °C with mild agitation. The resin was first washed with 6 ml of 1% SDS in PBS, and then 7 ml of 0.1 M urea in PBS followed by 10 ml of PBS. The resin underwent a final wash with 0.9 ml of 50 mM Tris-HCl and 1 mM CaCl<sub>2</sub> in water (pH 8.0) and then resuspended in 200 μl of 50 mM Tris-HCl, 1 mM CaCl<sub>2</sub> in water (pH 8.0), and 2 μg of porcine sequencing grade trypsin (Promega). Samples were digested overnight at 37 °C. Samples were then centrifuged at 2,000 × g for 4 min, and digest supernatants were decanted. Beads were washed in 100 μl of PBS centrifuged at 5,200 × g for 5 min, and the wash was combined with the digest supernatant. To the combined sample, trifluoroacetic acid (TFA) was added to 0.4% (v/v) or until pH <2. The sample was desalted with Oasis C18 10-mg columns (Waters) as described previously (66). The eluted sample was dried by speed vac and resuspended in 0.1 M HEPES buffer (pH 8.5). Samples were
labeled with Tandem Mass Tag™ 6-plex (TMTsixplex™) (Thermo Scientific) with the UV(+) sample labeled with TMT®- 128 or 131 reagent, the propofol protection sample labeled with TMT®- 126 or 129 reagent, and the UV(−) sample labeled with TMT®- 127 or 130 reagent using product instructions. Appropriate corresponding TMTsixplex™-labeled samples were pooled and dried by speed vac. The combined samples were resuspended in 0.5% acetic acid in water and pH-corrected with acetic acid until pH was <2. 40 μg of protein was desalted with C18 stage tips prepared in-house and dried by speed vac.

Samples were resuspended in 10 mM KH₂PO₄ (pH 2.6), 30% acetonitrile (v/v) in water, and fractionated by off-line strong cation exchange chromatography prior to mass spectrometry (MS) analysis similar to as reported previously (66). The full synaptosomal proteome control was prepared similarly without TMTsixplex™ labeling.

Mass Spectrometry Analysis—All TMT samples were analyzed with three-stage mass spectrometry (MS3) TMTsixplex™ quantification workflow as described previously (34). Spectral analysis was conducted using Thermo Proteome Discoverer 2.0 (Thermo Scientific) and mouse non-redundant (gene-centric) FASTA database. Mascot searches allowed for variable oxidation of methionine (+15.9949 m/z) and static modifications of cysteine residues (+57.0215 m/z; iodoacetamide alkylation) and TMTsixplex™ tags on lysine residues and peptide N termini (+229.162932 m/z). To establish the base synaptosomal proteome, searches allowed for variable oxidation of methionine (+15.9949 m/z) and static modifications of cysteine residues (+57.0215 m/z; iodoacetamide alkylation). All studies maintained trypsin enzyme specificity filtered with no greater than two missed cleavages. The MS2 spectral assignment was restricted to a specified false-positive rate of 1%, and a minimum of two unique peptides was required for protein identifications. Quantification was based on the theoretical m/z of the individual TMTsixplex™ reporter ions as reported previously (34). Enrichment factor was defined as the mean (+)UV/ (−)UV TMT ratio. Frequency distribution histograms of log2 values were generated using GraphPad Prism 7.0.

Western Blotting for Biotin-conjugated Protein Targets—750 μg of biotin-conjugated protein sample was resuspended via sonication in 500 μl of 25 mM NH₄HCO₃ and 6 μl urea in water. Following that, 150 μl of 5% Triton X-100 in water, 50 μl of 10% SDS in water, and 1.5 μl of 0.5 M DTT were added. The samples were heated for 15 min at 65 °C. Insoluble debris was separated by centrifugation for 10 min at 14,000 × g. The supernatant was diluted to 1 ml with PBS, and 50 μl was removed for the input sample. An additional 5 ml of PBS containing 100 μl of 50% streptavidin-agarose resin (Thermo Scientific) was added. Biotinylated proteins were captured over resin overnight at 4 °C with mild agitation. The resin was first washed with 6 ml of 1% SDS in PBS and then 7 ml of 0.1 M urea in PBS followed by 10 ml of PBS. The resin underwent a final wash with 0.9 ml of PBS and then was resuspended in 100 μl of 2 × SDS Laemmli buffer containing 100 mM DTT. Samples were then incubated with agitation at 37 °C for 30 min, centrifuged at 700 × g for 2 min, and heated for 15 min at 90 °C. 50 μl of 2 × SDS Laemmli buffer containing 100 mM DTT was joined to the input sample and heated for 5 min at 90 °C. Samples were centrifuged at 14,000 × g for 10 min prior to electrophoresis using 4–15% SDS-polyacrylamide gels with 10 μl of each sample introduced into each well. Proteins were then transferred to PVDF membranes. The membranes were blocked for 1 h with 2.5% BSA in Tris-buffered saline containing 0.1% Tween 20 (v/v; TBST). Membranes were incubated with GABA₆ receptor subunit antibodies overnight at 4 °C. All antibodies for GABA₆ receptor subunits were purchased from Santa Cruz Biotechnology, Inc., and included rabbit or goat polyclonal α1 ((A-20) sc-31405), α3 ((J-23) sc-122603), β1((N-19) sc-7361), β2 ((C-20) sc-7362), and γ2 ((Q-18) sc-101963) antibodies and monoclonal β3 (M-13 sc-376252) antibody. For GABA₆ receptor subunit analysis, membranes were washed three times with TBST prior to a 2-h incubation with appropriate HRP-conjugated secondary antibody at room temperature. All membranes were then washed twice with TBST and once with Tris-buffered saline (TBS) before being developed with Amersham Biosciences ECL select reagent and scanned. Only the net ratio of intensity-detected band(s) between 75 and 50 kDa was considered. The elution intensities were normalized to the corresponding input sample. Samples showing no detectable band elution were set to a net ratio of intensity of 0. Studies were conducted in quadruplicate and are represented as the fraction of the corresponding input.

Molecular Dynamics Simulations—A model of the α₁β₂γ₂ GABA₆ receptor was built by mutating 31 residues in the β subunits from an α₁β₂γ₂ GABA₆ model 3 reported in Hénin et al. (35). The mutations were made using the MUTATOR plugin of VMD (67). AutoDock Vina (68) was used to generate initial coordinates for propofol; default parameters were used, and the search space included the entire pentamer. AutoDock Vina returned at least one pose for each subunit interface; the ligand conformation with the best score was chosen for each site. The complex (GABA₆ receptor and five propofol molecules) was then placed in a 109 × 109 Å phosphatidylcholine membrane aligned parallel to the xy plane using CHARMM-GUI membrane builder (69). The system was solvated to a total height in z of 139 Å, followed by the addition of sodium and potassium ions that neutralized the system and brought the salt concentration to 0.15 M. The complete simulation system contained about 167,000 atoms.

The CHARMM36 force field was used for protein (70, 71) and phospholipid (72) parameters, with parameters for TIP3P waters (73) and ions (74) corresponding to those traditionally used with CHARMM-based force fields. Propofol parameters relied on atom types from CHARMM36, as described in LeBard et al. (75), further parameterization and use of a CMAP potential was required to accurately enforce coupling between rotation of the hydroxyl and isopropyl groups due to steric clashes.

Atomistic molecular dynamics simulations were run with NAMD version 2.10 (76). All simulations used periodic boundary conditions and particle mesh Ewald (PME) electrostatics. Interactions between non-bonded atoms were cut off at 12 Å, and bonds involving hydrogen were constrained using the SHAKE/RATTLE algorithm. A Langevin thermostat and barostat were used to maintain a temperature and pressure of 300 K and 1 atm, respectively, and vanishing surface tension was imposed. The simulation time step was 2 fs. Following the system generation, 30,000 minimization steps and a 7-ns
Propofol-binding Sites in Native Synaptic GABA<sub>A</sub> Receptor

equilibration protocol that gradually softened restraints on the protein and ligand were run. Subsequently, we ran a 200-ns production run with soft harmonic restraints on the Ca atoms (k = 0.5 kcal/mol Å<sup>2</sup>). The probability of hydrogen bonding was calculated using a VMD script that measured the fraction of frames in which propofol was hydrogen bonding to any residue in the site, detected using the VMD geometric criterion with a distance cutoff of 3.3 Å and an angle cutoff of 40°. The first 50 ns of the production run were not included in the analysis.

Binding affinities were calculated using the AFEP method, a theoretically exact method that involves gradually decoupling (reducing interaction strength) the ligand and the binding site throughout an MD simulation (44, 77). The decoupling free energy was then corrected by the ligand solvation free energy, as well as the entropic cost of transferring the ligand from the available volume per molecule in the standard state (1,660 Å<sup>3</sup>) to the volume of the ligand-binding site, yielding the standard Gibbs free energy of binding, ΔG<sup>0</sup>. The dissociation constant K<sub>D</sub> was calculated using the relationship K<sub>D</sub> = exp(−ΔG<sup>0</sup>/RT). Implementation of the method was very closely based on the procedure used by LeBard et al. (75) for propofol binding to intrasubunit site transmembrane domains of Gloeobacter ligand-gated ion channel. Decoupling of propofol from each of four interfaces was carried out in four separate simulations, over 24 windows, with 1 ns/window for a total of 24 ns per interfacing binding site.

The probability of propofol hydrogen bond formation (P<sub>HB</sub>) was estimated by calculating the frequency that a single hydrogen bond was formed between propofol and the propofol hydroxyl was detected over the course of the equilibrium MD simulation. Molecular images in Figs. 6, A and C–E, and 7 were generated using VMD (67), and the data in Fig. 6B was plotted and fit using python scripts.

Statistics—GraphPad Prism 7.0, ChemDraw Professional 15.0, and Microsoft Excel, unless otherwise noted, were used for figure preparation and statistical data analysis.

Supplemental Material—1<sup>1</sup>H, 13<sup>C</sup>, and 19<sup>F</sup> NMR spectra and detailed synthetic methods of AziP<sub>click</sub> (1) are presented in supplemental S2–S32. The identified propofol-specific proteome is presented in supplemental Table 1.

Author Contributions—K. A. W., X. W., B. A. G., J. H., G. B., S. M., R. S., R. E., M. C., and W. P. D. participated in research design. K. A. W., J. H., B. J. P., X. W., S. M., W. P. D., and R. S. conducted the experiments. X. W., B. A. G., G. B., R. S., S. M., J. H., W. P. D., and B. J. P. contributed new reagents or analytic tools. K. A. W., X. W., G. B., S. M., J. H., W. P. D., and R. S. performed data analysis. K. A. W., X. W., B. A. G., J. H., G. B., S. M., R. E., M. C., and W. P. D. wrote or contributed to the writing of the manuscript.

Acknowledgments—We thank Robert Pearce (University of Wisconsin) and Qiansheng Liang (Thomas Jefferson University) for GABA<sub>A</sub> receptor constructs and assistance with oocyte electrophysiology.

References
1. Olsen, R. W., and Sieghart, W. (2008) International union of pharmacology. LXX. Subtypes of γ-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. Pharmacol. Rev. 60, 243–260
2. Wu, X., Wu, Z., Ning, G., Guo, Y., Ali, R., Macdonald, R. L., De Blas, A. L., Luscher, B., and Chen, G. (2012) γ-Aminobutyric acid type A (GABA A) receptor α subunits play a direct role in synaptic versus extrasynaptic targeting. J. Biol. Chem. 287, 27417–27430
3. Hentschke, H., Benkwitz, C., Banks, M. I., Perkins, M. G., Homantics, G. E., and Pearce, R. A. (2009) Altered GABA<sub>A</sub>, slow inhibition and network oscillations in mice lacking the GABA<sub>A</sub> receptor β3 subunit. J. Neurophysiol. 102, 3643–3655
4. Kullmann, D. M., Ruiz, A., Rusakov, D. M., Scott, R., Semyanov, A., and Walker, M. C. (2005) Presynaptic, extrasynaptic and axonal GABA(A) receptors in the CNS: where and why? Prog. Biophys. Mol. Biol. 87, 33–46
5. Baumann, S. W., Baur, R., and Sigel, E. (2001) Subunit arrangement of γ-aminobutyric acid type A receptors. J. Biol. Chem. 276, 36272–36280
6. Baumann, S. W., Baur, R., and Sigel, E. (2002) Forced subunit assembly in α1β2/γ2 GABA<sub>A</sub> receptors. Insight into the absolute arrangement. J. Biol. Chem. 277, 46020–46025
7. Baur, R., Minier, F., and Sigel, E. (2006) A GABA(A) receptor of defined subunit composition and positioning: concatenation of five subunits. FEBS Lett. 580, 1616–1620
8. Luscher, B., Fuchs, T., and Kilpatrick, C. L. (2011) GABA(A)R trafficking-mediated plasticity of inhibitory synapses. Neuron 70, 385–409
9. Olsen, R. W., and Li G. D. (2011) GABA(A) receptors as molecular targets of general anesthetics: identification of binding sites provides clues to allosteric modulation. Can. J. Anaesth. 58, 206–215
10. Zecharia, A. Y., Nelson, L. E., Gent, T. C., Schumacher, M., Jurd, R., Rudolph, U., Brickley, S. G., Maze, M., and Franks, N. P. (2009) The involvement of hypothalamic sleep pathways in general anesthesia: testing the hypothesis using the GABA<sub>A</sub> receptor β3N265M knock-in mouse. J. Neurosci. 29, 2177–2187
11. Sanches-Segura, C., Cline, B., Jurd, R., Rudolph, U., and Spanagel, R. (2007) Etomidate and propofol-hyposensitive GABA<sub>A</sub> receptor γ2N236S mice show little changes in acute alcohol sensitivity but enhanced tolerance and withdrawal. Neurosci. Lett. 416, 275–278
12. Eckel, V. S., Rudolph, U., Antkowiak, B., and Grashoff, C. (2015) Propofol modulates phasic and tonic GABAergic currents in spinal ventral horn interneurons. Br. J. Anaesth. 114, 491–498
13. McDougall, S. J., Bailey, T. W., Mendelowitz, D., and Andresen, M. C. (2008) Propofol enhances both tonic and phasic inhibitory currents in second-order neurons of the solitary tract nucleus (NTS). Neuropharmacology 54, 552–563
14. Nishikawa, K. (2011) Roles of glutamatergic and GABAergic nervous system in hypnotic and analgesic actions of general anesthetics. Masui 60, 534–543
15. Chang, C. S., Olcese, R., and Olsen, R. W. (2003) A single M1 residue in the β2 subunit alters channel gating of GABA<sub>A</sub> receptor in anesthetic modulation and direct activation. J. Biol. Chem. 278, 42821–42828
16. Eaton, M. M., Cao, L. Q., Chen, Z., Franks, N. P., Evers, A. S., and Akk, G. (2015) Mutational analysis of the putative high-affinity propofol-binding site in human β3 homomeric GABA<sub>A</sub> receptors. Mol. Pharmacol. 88, 736–745
17. Krasowski, M. D., Nishikawa, K., Nikolaeva, N., Lin, A., and Harrison, N. L. (2001) Methionine 286 in transmembrane domain 3 of the GABA<sub>A</sub> receptor β subunit controls a binding cavity for propofol and other α-ketobenzyl alcohols. Neuropharmacology 41, 952–964
18. Jonsson Fagerlund, M., Sjödin, J., Krupp, J., and Dabrowski, M. A. (2010) Reduced effect of propofol at human GABA<sub>A</sub> receptor α1β2/γ2 mutant GABA(A) receptors. Br. J. Anaesth. 104, 472–481
19. Jayakar, S. S., Zhou, X., Chiara, D. C., Dostalova, Z., Savechenkov, P. Y., Bruzik, K. S., Dailey, W. P., Miller, K. W., Eckenhoff, R. G., and Cohen, J. B. (2014) Multiple propofol-binding sites in α-γ-aminobutyric acid type A receptor (GABA<sub>A</sub>) identified using a photoreactive propofol analog. J. Biol. Chem. 289, 27456–27468
20. Krasowski, M. D., Hong, X., Hopfinger, A. J., and Harrison, N. L. (2002) 4D-QSAR analysis of a set of propofol analogues: mapping binding sites

20484 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 291 • NUMBER 39 • SEPTEMBER 23, 2016 ASBMB
for an anesthetic phenol on the GABA(A) receptor. *J. Med. Chem.* **45**, 3210–3221

21. Krasowska, M. D., Jenkins, A., Flood, P., Kung, A. Y., Hopfinger, A. J., and Harrison, N. L. (2001) General anesthetic potencies of a series of propofol analogs correlate with potency for potentiation of γ-aminobutyric acid (GABA) current at the GABA(A) receptor but not with lipid solubility. *J. Pharmacol. Exp. Ther.* **297**, 338–351

22. Olsen, R. W. (2015) Allosteric ligands and their binding sites define γ-aminobutyric acid (GABA) type A receptor subtypes. *Adv. Pharmacol.* **73**, 167–202

23. Bertaccini, E. J., Yoluk, O., Lindahl, E. R., and Trudell, J. R. (2013) Assessment of homology templates and an anesthetic-binding site within the γ-aminobutyric acid receptor. *Anesthesiology* **119**, 1087–1095

24. León, I., Millán, J., Cocinero, E. J., Lesarri, A., Cañasto, F., and Fernández, J. A. (2012) Mimicking anesthetic-receptor interaction: a combined spectroscopic and computational study of propofol-phenol. *Phys. Chem. Chem. Phys.* **14**, 8956–8963

25. Jiang, W., Phillips, J. C., Huang, L., Fajer, M., Meng, Y., Gumbart, J. C., Luo, W., Weyermann, P., Gisselbrecht, J. P., Boudon, C., Diederich, F., and Gross, M. (1999) Dendritic iron porphyrins with tethered axial ligands: new model compounds for cytochromes. *Angew. Chem. Int. Ed. Engl.* **38**, 3215–3219

26. Breton, G. W. (1997) Selective monoacetylation of unsymmetrical diols catalyzed by silica gel-supported sodium hydrogen sulfite. *J. Org. Chem.* **62**, 8952–8954

27. Lea, W. A., Xi, J., Jadhav, A., Lu, L., Austin, C. P., Simeonov, A., and Eckenf, R. G. (2009) A high-throughput approach for identification of novel general anesthetics. *PLoS ONE* **4**, e7150

28. Hall, M. A., Xi, J., Lorr, C., Dai, S., Pearce, R., Dailey, W. P., and Eckenf, R. G. (2010) m-Azipropofol (Azilpm) a photoactive analogue of the intravenous general anesthetic propofol. *J. Med. Chem.* **53**, 5667–5675

29. McKinstry-Wu, A. R., Bu, W., Rui, G., Lea, W. A., Weiser, B. P., Liang, D. F., Simeonov, A., Jadhav, A., Maloney, D. J., and Eckenf, R. G. (2012) Discovery of Novel General Anesthetic Chemos-type Using High-throughput Screening. *Anesthesiology* **122**, 325–333

30. Vedula, L. S., Brannigan, G., Eronnou, N. I., Xi, J., Hall, M. A., Liu, R., Rossi, M. J., Dailey, W. P., Grasty, K. C., Klein, M. L., Eckenf, R. G., and Loll, P. J. (2009) A unitary anesthetic-binding site at high resolution. *J. Biol. Chem.* **284**, 24176–24184

31. Liu, R., Loll, P. J., and Eckenf, R. G. (2005) Structural basis for high-affinity volatile anesthetic binding in a natural 4-helix bundle protein. *FASEB J.* **19**, 567–576

32. Weiser, B. P., Kelz, M. B., and Eckenf, R. G. (2013) *In vivo* activation of azipropofol prolongs anesthesia and reveals synaptic targets. *J. Biol. Chem.* **288**, 1279–1285

33. Ting, L., Rad, R., Gygi, S. P., and Haas, W. (2011) MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. *Nat. Methods* **8**, 937–940

34. Hénin, J., Salari, R., Murlidaran, S., and Brannigan, G. (2014) A predicted binding site for cholesterol on the GABAA receptor. *Biophys. J.* **106**, 1938–1949

35. Wanzig, R. W. (1954) High-temperature equation of state by a perturbation method. 1. Nonpolar gases. *J. Chem. Phys.* **22**, 1420–1426

36. Hibbs, R. E., and Gouaux, E. (2011) Principles of activation and permeation in an anion-selective Cys-loop receptor. *Nature* **474**, 54–60

37. Miller, P. S., and Aricescu, A. R. (2014) Crystal structure of a human GABAA receptor. *Nature* **512**, 270–275

38. Westergard, T., Salari, R., Martin, J. V., and Brannigan, G. (2015) Correction: interactions of γ-3,5,3’,5’-triiodothyronine, allopregnanolone, and ivermectin with the GABA receptor: evidence for overlapping intersubunit binding modes. *PLoS ONE* **10**, e0142514

39. Woll, K. A., Weiser, B. P., Liang, Q., Meng, T., McKinstry-Wu, A., Pinch, B., Dailey, W. P., Gao, W. D., Covarrubias, M., and Eckenf, R. G. (2015) Role for the propofol hydroxy in anesthetic protein target molecular recognition. *ACS Chem. Neurosci.* **6**, 927–935
HCN1 channels as targets for anesthetic and nonanesthetic propofol analogs in the amelioration of mechanical and thermal hyperalgesia in a mouse model of neuropathic pain. *J. Pharmacol. Exp. Ther.* **345**, 363–373

61. Fassl, I., High, K. M., Stephenson, E. R., Yarotskyy, V., and Elmslie, K. S. (2011) The intravenous anesthetic propofol inhibits human L-type calcium channels by enhancing voltage-dependent inactivation. *J. Clin. Pharmacol.* **51**, 719–730

62. Barber, A. F., Liang, Q., and Covarrubias, M. (2012) Novel activation of voltage-gated K(+) channels by sevoflurane. *J. Biol. Chem.* **287**, 40425–40432

63. Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., Scalmani, G., Barone, V., Mennucci, B., Petersson, G. A., Nakatsuji, H., Caricato, M., Li, X., Hratchian, H. P., Izmaylov, A. F., et al. (2009) Gaussian 09, Revision D.01, Wallingford, CT

64. Butts, C. A., Xi, J., Brannigan, G., Saad, A. A., Venkatachalan, S. P., Pearce, R. A., Klein, M. L., Eckenhoff, R. G., and Dmochowski, I. J. (2009) Identification of a fluorescent general anesthetic, 1-aminoanthracene. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 6501–6506

65. Hirata, K., Akita, Y., Povalko, N., Nishioka, J., Yatsuga, S., Matsuishi, T., and Koga, Y. (2008) Effect of l-arginine on synaptosomal mitochondrial function. *Brain Dev.* **30**, 238–245

66. Villén, J., and Gygi, S. P. (2008) The SCX/IMAC enrichment approach for global phosphorylation analysis by mass spectrometry. *Nat. Protoc.* **3**, 1630–1638

67. Humphrey, W., Dalke, A., and Schulten, K. (1996) VMD: visual molecular dynamics. *J. Mol. Graph.* **14**, 27–28

68. Trotta, O., and Olson, A. J. (2010) Software news and update autodock vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **31**, 455–461

69. Jo, S., Lim, J. B., Klauda, J. B., and Im, W. (2009) CHARMM-GUI membrane builder for mixed bilayers and its application to yeast membranes. *Biophys. J.* **97**, 50–58

70. Best, R. B., Mittal, J., Feig, M., and MacKerell, A. D. (2012) Inclusion of many-body effects in the additive CHARMM protein CMAP potential results in enhanced cooperativity of α-helix and β-hairpin formation. *Biophys. J.* **103**, 1045–1051

71. MacKerell, A. D., Bashford, D., Bellott, M., Dunbrack, R. L., Evanseck, J. D., Field, M. J., Fischer, S., Gao, J., Guo, H., Ha, S., Joseph-McCarthy, D., Kuchnir, L., Kuczerka, K., Lau, F. T., Mattos, C., et al. (1998) All-atom empirical potential for molecular modeling and dynamics studies of proteins. *J. Phys. Chem. B.* **102**, 3586–3616

72. Klauda, J. B., Venable, R. M., Freites, J. A., O’Connor, J. W., Tobias, D. J., Mondragon-Ramirez, C., Vorobyov, I., MacKerell, A. D., Jr., and Pastor, R. W. (2010) Update of the CHARMM all-atom additive force field for lipids: Validation on six lipid types. *J. Phys. Chem. B.* **114**, 7830–7843

73. Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W., and Klein, M. L. (1983) Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* **79**, 926–935

74. Beglov, D., and Roux, B. (1994) Finite representation of an infinite bulk system: solvent boundary potential for computer simulations. *J. Chem. Phys.* **100**, 9050

75. LeBard, D. N., Hénin, J., Eckenhoff, R. G., Klein, M. L., and Brannigan, G. (2012) General anesthetics predicted to block the GLIC pore with micromolar affinity. *PLoS Comput. Biol.* **8**, e1002532

76. Philips, J. C., Braun, R., Wang, W., Gumbart, J., Tajkhorshid, E., Villa, E., Chipot, C., Skeel, R. D., Kalé, L., and Schulten, K. (2005) Scalable molecular dynamics with NAMD. *J. Comput. Chem.* **26**, 1781–1802

77. Gilson, M. K., Given, J. A., Bush, B. L., and McCammon, J. A. (1997) The statistical-thermodynamic basis for computation of binding affinities: a critical review. *Biophys. J.* **72**, 1047–1069