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Mutational analysis of the putative high-affinity propofol binding site in human β3 homomeric $GABA_A$ receptors

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Running title: Propofol actions in β3 homomers

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ABSTRACT

Propofol is a sedative and anesthetic agent that can both activate $GABA_A$ receptors and potentiate receptor activation elicited by submaximal concentrations of the transmitter. A recent modeling study of the β3 homomeric $GABA_A$ receptor (Franks, 2015, Anesthesiology 122:787) postulated a high-affinity propofol binding site in a hydrophobic pocket in the middle of a triangular cleft lined by the M1 and M2 membrane-spanning domains of one subunit and the M2 domain of the neighboring subunit. The goal of the present study was to gain functional evidence for the involvement of this pocket in the actions of propofol. Human β3 and α1β3 receptors were expressed in *Xenopus* oocytes, and the effects of substitutions of selected residues were probed on channel activation by propofol and pentobarbital. The data demonstrate a vital role of the β3(Y143), β3(F221), β3(Q224), and β3(T266) residues in the actions of propofol but not pentobarbital in β3 receptors. The effects of β3(Y143W) and β3(Q224W) on activation by propofol are likely steric because propofol analogs with less bulky *ortho* substituents activated both wild-type and mutant receptors. The T266W mutation removed activation by propofol in β3 homomeric receptors, however, this mutation alone or in combination with a homologous mutation (I271W) in the α1 subunit had almost no effect on activation properties in α1β3 heteromeric receptors. We hypothesize that heteromeric α1β3 receptors can be activated by propofol interactions with β3-β3, α1-β3 and β3-α1 interfaces but the exact locations of the binding site and/or nature of interactions vary in different classes of interfaces.

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INTRODUCTION

The $GABA_A$ receptor is the principal target of propofol and several other sedative and anxiolytic agents (Ferguson et al., 2007; Jurd et al., 2003; Low et al., 2000; Rudolph et al., 1999). Propofol directly activates the $GABA_A$ receptor and can potentiate currents elicited by a low concentration of the transmitter GABA (Hales and Lambert, 1991; Sanna et al., 1995). Under physiological conditions, this leads to hyperpolarization of cell or dampening of the effect of excitatory input.

Despite years of research, the location of the site(s) mediating the actions of propofol is unknown. A recent modeling study of the β3 homomeric receptor identified a hydrophobic cavity near the extracellular end of the membraneous region, predicted to bind propofol with a sub-micromolar equilibrium dissociation constant (Franks, 2015; Miller and Aricescu, 2014). The cavity lies in the center of a column triangle formed by the M1 and M2 membrane-spanning domains of the subunit contributing the "-" side of the interface (Chain A in Figure 1) and the M2 domain of the neighboring subunit that contributes the "+" side of the interface (Chain B in Figure 1). Molecular docking places the propofol molecule at the "-" side next to the β3(H267) residue (Franks, 2015). Incidentally, this residue is photolabeled by the photoreactive propofol analog, *ortho*-propofol diazirine (Yip et al., 2013). The key residue at the "+" side of the interface is T266 that points into the cleft towards the phenol ring of propofol. The cleft is capped from the extracellular side by the Y143 and F221 residues at the boundary between the extracellular and membrane-spanning domains (Franks, 2015).

Mutations to this region affect receptor properties. The β3(H267A) mutation shifts the GABA concentration-response curve of α1β3 receptors to higher agonist concentrations and reduces, albeit weakly, direct activation and potentiation of GABA-activated receptors by *ortho*-propofol diazirine (Yip et al., 2013). A tryptophan substitution at the β3(F221) site left shifts the GABA concentration-response curve, modestly reduces gating efficacy for *ortho*-propofol diazirine and

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essentially eliminates potentiation by this propofol analog. Overall, we consider the existing functional data supportive of the photolabeling and modeling data, but inconclusive, due to the relatively weak effect of the amino acid substitutions and absence of evidence that the effects are specific to the actions of propofol.

There are two caveats to prior functional data. The first concerns heterogeneity of intersubunit interfaces in the α1β3 receptor. The majority of studies now support the stoichiometry of two α subunits and three β subunits in αβ heteromeric receptors, assembled in the sequence of β-β-α-β-α (Baumann et al., 2001; Horenstein et al., 2001; Tretter et al., 1997). The α1β3 receptor thus has one β-β, two β-α, and two α-β interfaces. Mutations to the β3(H267) residue can be expected to modify and affect the β-β and α-β interfaces, but not the β-α interface, whose involvement in the actions of propofol has been suggested by previous photolabeling and functional studies (Bali and Akabas, 2004; Jayakar et al., 2014; Krasowski et al., 2001a). It is therefore plausible that contribution from the unaltered β-α interfaces conceals the true effect exerted by the H267A mutation at the "-" side of the β subunit. The second caveat is that the experiments were conducted using the photolabeling reagent *ortho*-propofol diazirine. It remained unclear whether the mutations influence activation by the parent compound, propofol.

Mutational studies in general can be ambiguous with regard to the underlying mechanism of effect. Changes in activation properties may result from the mutation interfering with the binding of ligand or signal transduction, with the distinction between the two not always being straightforward (Colquhoun, 1998). Photolabeling studies may present a more direct approach to identify the regions involved, however, a mutational study can reveal the functional involvement of a site.

To gain further insight into the role of the cavity formed by the M1 and M2 membrane-spanning domains at the intersubunit interface, we tested the effects of mutations to selected residues in β3 homomeric receptors. The data show that tryptophan substitutions at β3(Y143),

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β3(F221), β3(Q224), and β3(T266) drastically reduce activation by propofol with relatively modest effects on activation by pentobarbital. The effects of mutations were dependent on the *ortho* substituents of the phenol backbone, implying an underlying steric nature. The β3(H267W) and the neighboring β3(L268W) mutations had minimal effect on receptor activation by propofol or pentobarbital. Comparison of the effects of β3(T266W) and the homologous mutation in the α1 subunit (α1(I271W)) suggests that propofol-receptor interactions at the α1-β3 and β3-α1 interfaces are not equivalent with regard to the structures involved.

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MATERIALS AND METHODS

The experiments were conducted on wild-type and mutant human β3 and α1β3 $GABA_A$ receptors, expressed in *Xenopus* oocytes. Harvesting of oocytes was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The protocol was approved by the Animal Studies Committee of the Washington University in St. Louis.

The mutations employed in the study were: Y143W, F221W, Q224W, T263W, T266W, H267W, and L268W in the β3 subunit, and I271W in the α1 subunit. The β3(H267) residue was recently identified as a component of the propofol binding pocket using photolabeling with the propofol analogue *ortho*-propofol diazirine (Yip et al., 2013). The β3(Y143), β3(F221), β3(Q224), β3(T263), β3(T266), β3(L268), and α1(I271) residues are located around the perimeter of the putative propofol binding pocket (Figure 1). Although this putative binding pocket was identified (Franks, 2015) using a relatively low-resolution (3Å) crystal structure (Miller and Aricescu, 2014), this was quite sufficient to highlight which residues might contribute to a possible propofol binding site.

All mutations were made using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The β3 subunit contained the FLAG epitope in the amino terminus of the subunit (Ueno et al., 1996). The clones were fully sequenced prior to use. All mutated receptors, with the exception of β3(T263W) homomers, were functional.

The cDNAs, subcloned into the pcDNA3 vector, were linearized by digestion with Xba I (β3; NEB Labs, Ipswich, MA) or Bgl II (α1; Roche Diagnostics, Indianapolis, IN). The cRNAs were produced using mMessage mMachine (Ambion, Austin, TX). The oocytes were injected with a total of 3-18 ng cRNA in a final volume of 20-40 nl, and incubated in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM Na pyruvate, 5 mM HEPES; pH 7.4) at 16 ^oC. The

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ratio of cRNAs used for injection was 5:1 when α1 and β3 subunits were used, to reduce the fraction of β3 homomeric receptors. The oocytes were used within 1-3 days after injection.

Electrophysiological experiments were conducted using the two-electrode voltage clamp technique. Voltage and current electrodes were patch-clamp electrodes that when filled with 3 M KCl had resistances of less than 1 MΩ. The oocytes were clamped at -60 mV. The chamber (RC-1Z, Warner Instruments, Hamden, CT) was perfused continuously at approximately 5 ml min^{-1}. Bath solution (92.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES; pH 7.4) was perfused between all test applications. Solutions were gravity-applied from 30-ml glass syringes with glass luer slips via Teflon tubing to reduce adsorption, and switched manually or by pClamp using a Warner Instruments VC-8T valve controller. A typical recording consisted of a 10-s baseline followed by a 10-60 s drug application and a bath application (up to 10 min) until full recovery. The duration of drug (propofol, pentobarbital, picrotoxin, or GABA) application was dependent on the nature of drug and its concentration, and was aimed at reaching a saturated peak response without unnecessary further exposure to the drug, to facilitate washout and avoid accumulation of the drug in the cell. The current responses were amplified with an Axoclamp 900A amplifier (Molecular Devices, Sunnyvale, CA), digitized with a Digidata 1320 series digitizer (Molecular Devices) at a 100 Hz sampling rate, and stored using pClamp (Molecular Devices). The traces were subsequently analyzed with Clampfit (Molecular Devices) to determine the maximal amplitude of current response.

Concentration-response curves were fitted, individually for each cell, with the following equation:

$$Y=Y_{max}*([drug]^{nH}/([drug]^{nH}+EC_{50}{}^{nH})) \qquad \text{(Eq. 1)}$$

where EC_{50} is the concentration of drug producing a half-maximal effect, n_H describes the slope of relationship, and Y_{max} is the high concentration asymptote. At high concentrations, both

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propofol (Adodra and Hales, 1995) and pentobarbital (Akaike et al., 1987) block current responses. This manifests as a suppressed initial peak response followed by a prominent tail or rebound response following termination of drug application. The rebound response reflects transient repopulation of the conducting state(s) of the channel that occurs during washout of the drug. We used the amplitude of the rebound response in curve fitting whenever it was greater than the initial response. Fitting was conducted using the NFIT software (The University of Texas, Medical Branch at Galveston, Galveston, TX). Parameters of the fit are reported as mean ± S.E.M. Statistical analysis was conducted using two-sample t-test (Excel, Microsoft, Redmond, WA).

The level of spontaneous activity was determined by comparing the effect of 10-100 μM picrotoxin to the response to saturating pentobarbital which, in all cases where β3 homomeric configuration was employed, produced a larger current response than saturating propofol. Spontaneous activity, expressed in units of estimated open probability (P_o^{est}), was calculated assuming that P_o was 0 in the presence of saturating picrotoxin and 1 in the presence of saturating pentobarbital. This approach is similar to the one described previously for heteromeric $GABA_A$ receptors (Eaton et al., 2014; Forman and Stewart, 2012). We note, however, that this approach may result in overestimation of $P_{o,spont}$, because the actual P_o in the presence of saturating pentobarbital may be less than 1. In some cases (e.g., β3(F221W)), saturating pentobarbital was more effective at blocking spontaneous activity than 100 μM picrotoxin. For those receptors, $P_{o,spont}^{est}$ was calculated assuming that the P_o reached 0 during the initial blocking action of pentobarbital and 1 during the rebound response following termination of pentobarbital application.

Parameters for binding and gating in the presence of propofol or pentobarbital were derived from fitting the P_o^{est} from pooled data to the following equation (Chang and Weiss, 1999; Rusch et al., 2004):

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$$P_o^{est}=(1+L_o(1+[agonist]/K_C)/(1+[agonist]/dK_C))^n)^{-1} \quad \text{(Eq. 2)}$$

where L_o is the ratio of the equilibrium occupancy of closed receptors to the equilibrium occupancy of open receptors in the absence of agonist, K_C stands for the closed receptor equilibrium dissociation constant for given agonist, *d* is a measure of efficacy expressed as the ratio of open receptor dissociation constant to closed receptor dissociation constant, and n is an integer, constrained to 2 to 5, corresponding to the number of agonist binding sites occupied to produce activation.

Fitting was conducted using NFIT. The fitting results shown below were obtained with n of 5 for propofol and 4 for pentobarbital. We note that changes in n had a relatively weak effect on the goodness of the fit. K_C and *d* were free parameters. L_o was experimentally determined as $(1-P_{o,spont}^{est})/P_{o,spont}^{est}$, and equaled to 6.7, 13.3, 99, 0.7, 3.8, or 1.9 in β3 wild-type, β3(H267W), β3(L268W), β3(F221W), β3(Y148W), and β3(T266W), respectively. The β3(Q224W) receptor showed no picrotoxin-sensitive spontaneous activity. To estimate the parameters for binding and gating in this mutant, we constrained L_o to arbitrarily chosen values of 100, 1000, 10000 and 100000 (corresponding to $P_{o,spont}$ of approximately 0.01, 0.001, 0.0001 and 0.00001, respectively). The best fit was obtained with L_o of 1000. The results of the fit are reported in Table 2.

Potentiation of α1β3 receptors was estimated by examining the effect of a low concentration of propofol on currents elicited by a low concentration of GABA. Test GABA concentration was selected to produce a response of approximately 5% of the maximal response to GABA. Propofol was used at a concentration that elicited a response of less than 3% of the maximal response to GABA. The potentiating effect of propofol was calculated as $I_{(GABA+propofol)} / (I_{GABA} + I_{propofol})$. Statistical analysis was conducted by comparing the potentiating effect to 1 (i.e., no effect), using a two-tailed paired t-test (Excel). This test, equivalent to a one-sample t-test with a

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hypothetical value of 1, is designed to determine whether the effect of propofol is statistically significant.

Pentobarbital, propofol analogs, and inorganic salts used in buffers were bought from Sigma-Aldrich (St. Louis, MO). Propofol was from MP Biomedicals (Solon, OH). Stock solution of 5 mM pentobarbital was made in the bath solution. Stock solutions of 10 or 200 mM propofol or analogs were made in DMSO. Stock solutions were kept at room temperature and further diluted as needed on the day of the experiment.

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RESULTS

Activation of wild-type and mutant human β3 homomeric receptors by propofol and pentobarbital

It was recently shown that *ortho*-propofol diazirine, a propofol analog photolabeling reagent, labels the β3(H267) residue in human β3 and α1β3 $GABA_A$ receptors expressed in Sf9 cells (Yip et al., 2013). The β3(H267) residue is located near the extracellular end (by convention, the 17' residue) of the second membrane-spanning domain, pointing to the interior of the subunit where it flanks a hydrophobic cavity located between the TM2 and TM1 domains of two neighboring β subunits (Franks, 2015; Yip et al., 2013). Previous work has indicated a potential involvement of the 267 location in the actions of Zn^{2+} (Dunne et al., 2002; Trudell et al., 2008) and picrotoxinin (Carland et al., 2008). We mutated the β3(H267) residue to tryptophan. This substitution results in a more than 50% increase in bulkiness (a ratio of volume to length) of the sidechain (Zimmerman et al., 1968).

Our data indicate that the H267W mutation does not affect propofol-activation of β3 receptors. The midpoint of the propofol concentration-response curve was at 9 ± 1 μM (mean ± S.E.M.; 7 cells) in wild-type β3 receptors and at 10 ± 1 μM (8 cells) in β3(H267W). We also probed the effect of the mutation on activation by pentobarbital. In four cells expressing wild-type β3 receptors, the EC_{50} for pentobarbital was 59 ± 7 μM. In receptors containing the β3(H267W) mutation, the pentobarbital EC_{50} was 67 ± 20 μM (4 cells). The difference was not statistically significant. To gain insight into the effect of β3(H267W) on gating efficacy, we compared responses to saturating (30-100 μM) propofol and saturating (300-1000 μM) pentobarbital. In wild-type β3 receptors, propofol elicited a response that was 53 ± 3% of the response to pentobarbital (8 cells). In β3(H267W) receptors, the response to propofol was 27 ± 1% of the response to pentobarbital (5 cells). The difference was statistically significant ($p < 0.001$; t-test; Excel, Microsoft, Redmond, WA). We also examined the extent of spontaneous

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activity in wild-type β3 and β3(H267W). By comparing the effect of 10-100 μM picrotoxin on baseline current to the peak response to 300 μM pentobarbital, we calculate (see Methods for details and caveats) that $P_{o,spont}^{est}$ is 0.13 ± 0.02 (8 cells) in wild-type and 0.07 ± 0.02 in β3(H267W) (5 cells; $p > 0.05$). The data are summarized in Figure 2 and Table 1. Fitting equation 2 to pooled P_o^{est} data yielded a K_C of 8.8 μM and the parameter *d* (K_O/K_C) of 0.64 for wild-type β3 receptors and 7.1 μM and 0.67 for β3(H267W) (Figure 3, Table 2). We infer based on these data that the histidine sidechain in position 267 does not contribute significantly to activation properties of β3 homomeric $GABA_A$ receptors.

We conducted further mutational analysis of selected residues in the putative propofol binding pocket. The β3(L268W) mutation had a minor influence over activation properties. The propofol activation curve had an EC_{50} of 15 ± 2 μM (4 cells). The concentration-response curve for activation by pentobarbital was right-shifted and had an EC_{50} of 272 ± 40 μM (6 cells). The mean peak current in the presence of saturating (100 μM) propofol was 14 ± 1% (5 cells) of the response to saturating (5 mM) pentobarbital. Application of up to 100 μM picrotoxin had minimal (<1% of the maximal pentobarbital response; 5 cells) effect on the holding current, indicating a low level of spontaneous activity. The fitted K_C and *d* for propofol were essentially unchanged (6.5 μM and 0.55) compared to wild-type.

Franks (Franks, 2015) proposed that the main chain carbonyl oxygen of residue β3(Q224) forms a hydrogen bond with the oxygen atom of propofol. While we were unable to make modifications to the peptide bond, we reasoned that addition of bulk to the sidechain at this position may interfere with optimal positioning of the propofol molecule in the cavity and affect propofol activation properties. Receptors containing the β3(Q224W) mutation were essentially not responsive to propofol. Application of 500 μM propofol (a concentration over 50-fold higher than EC_{50} in β3 wild-type) elicited a response that was only 2.7 ± 0.2% (4 cells) of the response to saturating (3 mM) pentobarbital. We also probed activation in the presence of 50 μM propofol. In 6 cells, the peak response was 3.0 ± 0.4% of the response to 3 mM pentobarbital. While the

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currents elicited by propofol are small and the response at 500 μM propofol may be influenced by block, these data suggest that the β3(Q224W) mutation may suppress gating rather than binding of propofol. The mutation also affected pentobarbital concentration-response relationship. The pentobarbital activation curve had a midpoint at 666 ± 21 μM (4 cells). Lack of effect of picrotoxin (3 cells) on the holding current and inability of blocking concentrations of pentobarbital to elicit apparent outward current indicates minimal spontaneous activity in β3(Q224W).

The putative propofol-binding cavity is capped at the extracellular side by β3(Y143) and β3(F221) (Figure 1). We probed the functional effects of tryptophan substitutions at these locations. In oocytes expressing β3(F221W) homomeric receptors, exposure to 20 μM propofol was without effect (7 cells) whereas application of 500 μM propofol elicited an apparent outward current (3 cells), indicative of block of spontaneous activity. Exposure to 10-100 μM picrotoxin also resulted in apparent outward current. Application of 3 mM pentobarbital resulted in an initial outward current that we interpret as block of spontaneous activity, followed by an inward rebound current upon the removal of the drug, that extended beyond the baseline level (a total of 22 cells). Incidentally, pentobarbital, at 3 mM, was a more efficacious blocker than 100 μM picrotoxin or 500 μM propofol. These findings indicate that the β3(F221W) mutant produces a high level of spontaneous activity. Using the current level during the application of 3 mM pentobarbital for P_o^{est} of 0 and the rebound current level in the end of pentobarbital application for P_o^{est} of 1, we estimate that $P_{o,spont}^{est}$ equals 0.62 ± 0.04 (22 cells). The concentration-response relationship for pentobarbital was rightshifted in the mutant and had an EC_{50} of 523 ± 45 μM (9 cells).

The propofol activation curve in β3(Y143W) had an EC_{50} of 22 ± 6 μM (5 cells; $p < 0.05$ vs. wild-type). The concentration-response relationship for pentobarbital was not affected by the mutation, and had an EC_{50} of 82 ± 24 μM (5 cells). We estimate that the $P_{o,spont}^{est}$ for β3(Y143W) is 0.21 ± 0.02 (4 cells), which is a moderate increase ($p < 0.05$) over $P_{o,spont}^{est}$ in wild-type. By

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comparing maximal currents in the presence of propofol and pentobarbital, we estimate that the maximal P_o^{est} in the presence of propofol is only 0.3 ± 0.05 (4 cells). Fitting pooled data to equation 2 yielded a K_C of 14.4 μM and a *d* (K_O/K_C) of 0.91.

We also tested the effect of placing a tryptophan residue in place of β3(T266). This residue is pointed towards the putative propofol-binding cavity, however, it is supplied by the neighboring subunit that contributes the "+" side to the intersubunit interface (Figure 1). The β3(T266W) receptor showed minimal activation by propofol. In the presence of 10 μM propofol, the peak response was 4 ± 1% (7 cells) of the response to 3 mM pentobarbital. Exposure to higher concentrations of propofol resulted in outward current that we interpret as block of spontaneous activity. The concentration-response relationship for pentobarbital was shifted to higher agonist concentrations (EC_{50} = 389 ± 81 μM; 4 cells). By comparing block by 100 μM picrotoxin to the rebound response upon termination of the application of saturating (3 mM) pentobarbital we estimate that the open probability of spontaneous activity is 0.34 ± 0.08 (5 cells) in β3(T266W).

Activation of wild-type and mutant β3 receptors by propofol analogs

To gain insight into the mechanism by which tryptophan substitutions reduce activation by propofol, we examined the effects of mutations on activation by propofol analogs with different *ortho* substituents. The experiments were conducted by comparing responses to a saturating concentration of pentobarbital (300 μM in β3 wild-type, 3 mM in mutants) and, typically, 500 μM propofol analog in the same cell. In some cases (e.g., 2-*tert*-butyl-6-methylphenol on F221W or T266W), only blockade of spontaneous activity was observed at 500 μM, so a lower concentration (10 μM) was employed to compare peak responses.

The data suggest that the deleterious effect of the β3(Y143W) mutation on propofol activation has a steric origin. Activation by compounds with compact *ortho* substituents (2,6-

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dimethylphenol) or a single *ortho* substituent (2-isopropylphenol) was not affected by the β3(Y143W) mutation. The ratio of responses to 2,6-dimethylphenol over pentobarbital was 0.74 ± 0.17 (4 cells) in the mutant and 0.77 ± 0.07 (4 cells) in the wild-type receptor. The relative response to 2-isopropylphenol was 1.44 ± 0.19 (4 cells) in the mutant and 1.56 ± 0.25 (4 cells) in the wild-type receptor. A compound with a relatively bulky *ortho* substituent (2,6-diethylphenol) was significantly worse at activating the mutant than wild-type receptors. The ratio of responses to 2,6-diethylphenol over pentobarbital was 0.14 ± 0.03 (4 cells) and 0.78 ± 0.03 (4 cells) in β3(Y143W) and β3 wild-type, respectively. The β3(Y143W) mutation also reduced the relative current in the presence of 2-*tert*-butyl-6-methylphenol, that elicited a relative response of 0.53 ± 0.10 (5 cells) in the wild-type and 0.25 ± 0.04 (4 cells) in the mutant receptor.

The β3(Q224W) mutation diminished gating by 2,6-dimethylphenol (response ratio 0.29 ± 0.01; 4 cells) and 2,6-diethylphenol (0.14 ± 0.03; 4 cells) but not 2-isopropylphenol (1.37 ± 0.11; 4 cells) or 2-*tert*-butyl-6-methylphenol (0.66 ± 0.07; 4 cells). This indicates an asymmetrical requirement for one bulky and one compact or missing *ortho* substituent.

Receptors containing the β3(F221W) or β3(T266W) mutation showed strongly diminished responses in the presence of all tested propofol analogs. In β3(F221W), the response ratios were 0.16 ± 0.03 (5 cells), 0.03 ± 0.01 (5 cells), 0.01 ± 0.01 (3 cells), and 0.02 ± 0.02 (3 cells) for 2-isopropylphenol, 2,6-dimethylphenol, 2,6-diethylphenol, and 2-*tert*-butyl-6-methylphenol, respectively. When the receptor contained the β3(T266W) mutation, the response ratios were 0.12 ± 0.03 (5 cells), 0.13 ± 0.04 (6 cells), 0.12 ± 0.02 (5 cells), and 0.06 ± 0.02 (4 cells) for 2-isopropylphenol, 2,6-dimethylphenol, 2,6-diethylphenol, and 2-*tert*-butyl-6-methylphenol, respectively. We propose that these residues are involved in signal transduction or located near an unaltered part of the propofol molecule (e.g., the hydroxyl group). The data are summarized in Figure 4.

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Properties of α1β3 receptors containing the β3(T266W) or α1(I271W) mutation

In heteromeric α1β3 receptors, the residue homologous to β3(T266), supplied by the "+" side of the interface, is α1(I271). We examined the effect of the α1(I271W) mutation on properties of α1β3. Our expectation was that this mutation imitates the effect of β3(T266W) in β3 homomeric receptors. However, α1(I271W)β3 receptors behaved, in most aspects, similar to wild-type α1β3 receptors. The mutation weakly, but statistically significantly ($p < 0.05$; t-test), left-shifted the propofol concentration-response curve. The propofol EC_{50} was 13 ± 2 μM (6 cells) in wild-type, and 5 ± 1 μM (4 cells) in the mutant. The concentration-response relationship for pentobarbital was not affected by α1(I271W). The EC_{50}s were 87 ± 13 μM (5 cells) and 68 ± 5 μM (4 cells) in wild-type and mutant, respectively. Unlike β3 homomeric receptors, the α1β3 receptors are activated by GABA. We found that the GABA concentration-response curve was modestly left-shifted in α1(I271W)β3. The midpoint of the curve was at 0.6 ± 0.1 μM (4 cells) in the mutant and at 1.4 ± 0.1 μM (4 cells) in wild-type ($p < 0.01$). The data are summarized in Figure 5 and Table 3.

We also conducted a test on receptor potentiation by propofol. For that, GABA at a concentration producing an EC_5 response was applied in the absence and presence of a low concentration of propofol (see Methods for details). Proper comparison of potentiation across mutants may be complicated by differences in maximal open probability for GABA, so we used this experiment as a simple qualitative test of whether potentiation is present or absent in the particular receptor.

As expected, wild-type α1β3 receptors were potentiated by propofol. Co-application of 1 μM propofol, that by itself elicited a response with a peak of <1% of the response to saturating GABA, with 0.3 μM GABA (EC_5) resulted in 5 ± 1 fold potentiation (5 cells). In α1(I271W)β3, co-application of 0.5 μM propofol with 0.1 μM GABA led to 3 ± 0.1 fold (6 cells) potentiation of the current response. Overall, the data demonstrate that the α1(I271W) mutation has minimal effect

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on activation and modulation of α1β3 receptors.

The α1(I271W) mutation is expected to influence propofol actions at the α(+)-β(-) interface. However, previous work has demonstrated that propofol (and other anesthetic drugs) may act via the β(+)-α(-) interface (Bali and Akabas, 2004; Jayakar et al., 2014; Krasowski et al., 2001a; Li et al., 2006), that remained unmodified in α1(I271W)β3 receptors. To additionally introduce the tryptophan substitution to the β-α interface, we combined α1(I271W) and β3(T266W) subunits. This double mutant receptor contains the tryptophan residue at homologous sites at "+" sides of α-β and β-α, and β-β interfaces, and can be considered a conceptual analog of the β3(T266W) homomeric receptor. To our surprise, the double mutation had minimal influence on channel properties. The propofol EC_{50} was not affected (9 ± 1 μM, 8 cells vs. 13 μM in wild-type, $p > 0.05$). The concentration-response relationship for pentobarbital was unaffected (EC_{50} was 113 ± 30 μM; 4 cells). The activation curve for GABA was right-shifted by approximately 2-fold (midpoint at 3.3 ± 0.7 μM; 4 cells; $p < 0.05$). The α1(I271W)β3(T266W) receptors were potentiated by propofol. Currents elicited by 0.1 μM GABA (EC_4) were enhanced 3 ± 0.1 fold (6 cells) in the presence of 1 μM propofol.

As a negative control, we examined the properties of the α1β3(T266W) receptor. As expected, this mutation had a relatively small effect on activation and potentiation properties. The midpoint of the propofol concentration-response curve was left-shifted to 4.0 ± 0.5 μM (5 cells; $p < 0.01$). In the presence of pentobarbital, there was a trend in EC_{50} to higher concentrations (EC_{50} = 166 ± 42 μM, 5 cells), but the effect did not reach statistical significance. The EC_{50} for GABA was left-shifted to 0.2 ± 0.04 μM (4 cells). Receptors activated by low GABA (EC_5) were potentiated by 3.5 ± 0.2 fold (5 cells) in the presence of 1 μM propofol.

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DISCUSSION

A recent modeling study postulated a high-affinity propofol binding site at the intersubunit interface in β3 homomeric $GABA_A$ receptors (Franks, 2015). The putative binding site lies in the middle of a triangular structure formed by the M1 and M2 membrane-spanning domains of a subunit contributing the "-" side of the interface and the M2 domain of the neighboring subunit that contributes the "+" side of the interface (Figure 1). The goal of the present study was to investigate the role of selected residues lining this cavity, using electrophysiology to gain functional evidence for its role in activation by propofol. We introduced tryptophan residues to Y143, F221, Q224, and H267 at the "-" side and T266 (in the β3 subunit) or I271 (α1 subunit) at the "+" side of the intersubunit interface. The major experimental finding is that mutations at some of these locations strongly reduce or eliminate activation by propofol with less significant effect on activation by pentobarbital.

We began this study with the null hypothesis that the cavity around the β3(H267) residue is not involved in activation of the β3 homomeric $GABA_A$ receptor by propofol. We reasoned that while a functional effect of a mutation is supportive of involvement but ultimately inconclusive, lack of selective functional effect is an indication that a residue is not a critical component of the interaction site. We chose to make tryptophan substitutions. Tryptophan, due to its added bulkiness, is more likely to exclude the ligand, or, perhaps, mimic its presence in the binding pocket. Previous studies examining properties of putative binding sites for neurosteroids and the anesthetic etomidate have found that tryptophan substitution of key residues can mimic the effects of a bound modulator, such as increased spontaneous activity and left-shifted GABA concentration-response curves (Akk et al., 2008; Stewart et al., 2008).

Even though the β3(H267) residue is labeled by the propofol analog *ortho*-propofol diazirine (Yip et al., 2013) and is located adjacent to the modeled high-affinity propofol binding site (Franks, 2015), a tryptophan substitution at this position had minimal influence on activation

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properties. Specifically, there was no increase in spontaneous activity or shift in propofol EC_{50}. Comparison of maximal currents in the presence of propofol and pentobarbital in β3(H267W) suggests a nearly 2-fold reduction in gating efficacy by propofol, but the caveat is that the difference may be caused by a change in P_o for pentobarbital rather than propofol. In the case of heteromeric $GABA_A$ receptors, the estimate for maximal P_o can be obtained by examining the ability of various potentiators to enhance the response to saturating agonist (e.g., (Eaton et al., 2014; Forman and Stewart, 2012)). We were, however, unable to observe potentiation of β3 homomeric receptors activated by pentobarbital in the presence of propofol or the steroid alphaxalone (not shown). Our findings are in agreement with a previous work that found a two-fold reduction in direct activation by the propofol analog *ortho*-propofol diazirine in α1β3 receptors containing the β3(H267A) mutation (Yip et al., 2013).

While photolabeling of H267 with *ortho*-propofol diazirine was the aegis for identifying the potential binding pocket at the top of β3-TM2, it appears not to be directly involved in propofol binding or effect. This may be due to its mechanism of photolabeling. Following photolysis of *ortho*-propofol diazirine, an *ortho*-quinone methide would be predicted as a major photo product. Quinone methides have relatively long half-lives (Silva and Bozzelli, 2007) and, as strong electrophiles, will preferentially react with nearby nucleophilic amino acids (Modica et al., 2001). There are numerous water accessible nucleophilic amino acids in the β3 homomers (Miller and Aricescu, 2014). The fact that nucleophilic amino acids other than β3(H267) are not labeled suggests that *ortho*-propofol diazirine binding is concentrated near β3(H267) or may diffuse a short distance to photolabel it.

Mutations at the top of the putative binding cavity, near the boundary between the membrane-spanning and extracellular domains, had a major effect on activation by propofol. No propofol-elicited currents were observed in β3(F221W), and this mutation resulted in large (P_o of 0.62) spontaneous currents. A tryptophan substitution at the nearby β3(Y143) residue had a modest effect on propofol affinity, but strongly reduced gating efficacy. At the β3(Q224) site, a

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tryptophan substitution essentially eliminated activation by propofol. The F221W, Y143W, and Q224W mutations had a relatively modest effect on activation of the β3 receptor by pentobarbital. We infer that the region between the membrane-spanning and extracellular domains occupied by the F221, Y143, and Q224 residues is a key determinant of activation by propofol.

Near the cytoplasmic end of the putative propofol binding site, we examined the effects of tryptophan substitutions at the β3(T263) and β3(T266) sites. The β3(T263W) receptor was not functional in the presence of propofol or pentobarbital at up to 1 mM (not shown). Application of 100 μM picrotoxin had no effect on the holding current indicating the absence of spontaneous activity (not shown). The β3(T266W) receptor showed increased spontaneous activity, a right-shifted pentobarbital concentration-response relationship, but minimal activity in the presence of propofol.

Fitting P_o^{est} data to Equation 2 revealed some unexpected information. The fitted gating efficacy parameter (*d*) indicated a surprisingly small difference in the affinity of closed and open β3 receptors to propofol. In wild-type β3, β3(H267W), and β3(L268W), propofol binds only ~2 times (d^{-1}) more tightly to open receptors than to closed receptors. This corresponds to a stabilization energy of 2 kcal/mol (assuming 5 binding sites). To put this in perspective, the estimate for d^{-1} for propofol in heteromeric α1β2γ2L receptors is 50, that corresponds to a stabilization energy of 6.9 kcal/mol in a receptor with three binding sites (Ruesch et al., 2012). In β3(Y143W), d^{-1} was 1.1 and the stabilization energy 0.3 kcal/mol. For pentobarbital, d^{-1} was 14, 50, and 3, corresponding to stabilization energies of 6.3, 9.2, and 2.5 kcal/mol in wild-type β3, β3(H267W), and β3(Y143W), respectively.

Equation 2 is derived from an allosteric activation model (Figure 3) where the receptor can exist in multiple states with different affinities to ligand (Colquhoun, 1998; Monod et al., 1965). In this framework, β3(H267W) and β3(L268W), that show apparent decreased gating efficacy in the absence (L_o) and presence (dL_o) of propofol but an unchanged K_O/K_C (*d*) for propofol, have

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a pure, albeit weak, gating effect. The β3(Y143W) with *d* of 0.9 and a small effect on K_C, predominantly affects the equilibrium dissociation constant of open receptors suggesting that this residue may come in contact with the propofol molecule in the active state where the tryptophan side chain results in unfavorable interaction with the agonist. The deleterious effect of the mutation likely has a steric origin because propofol analogues 2,6-dimethylphenol and 2-isopropylphenol were efficacious activators of the mutant receptor. Two other mutations (F221W and T266W) in β3 receptors exhibited large $P_{o,spont}^{est}$ but no response to propofol. This can be accounted for by unchanged equilibrium dissociation constants in closed and open mutant receptors, i.e., *d* near unity. We note that there was a wide range of effects on $P_{o,spont}^{est}$ in mutant receptors, which perhaps is not surprising given the transmembrane location of most locations. We were, however, unable to find good correlation between changes in $P_{o,spont}^{est}$ and effects on activation by propofol.

Overall, our mutational analysis strongly supports, although does not prove, the involvement of the cleft defined by the M2 and M1 membrane-spanning domains from neighboring subunits in the actions of propofol in β3 $GABA_A$ receptors. We have identified four residues (Y143, F221, Q224, and T266) where a tryptophan substitution strongly reduced activation or rendered the receptor inactive in the presence of propofol but not pentobarbital. Work with propofol analogs showing that the magnitude of effects of mutations is dependent on *ortho* substituents of propofol supports the notion that these residues are components of propofol binding site in the β3 receptor.

We employed β3 homomeric receptors to avoid complications arising from heterogeneity in subunit interfaces. The α1β3 receptor is expected to contain one β-β, two α-β, and two β-α interfaces. If we assume that mutations to the "-" side of β3 affect both β-β and α-β type interfaces that still leaves two unmodified β-α interfaces. A recent study that employed the photoreactive propofol analog 2-isopropyl-5-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenol demonstrated labeling at the β-α interface in human α1β3 receptors (Jayakar et al., 2014).

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Interestingly, the residues labeled (β3(M286), α1(M236), and α1(I239)) are located in a plane more cytoplasmic relative to the key residues in β3 homomers in the long axis of membrane-spanning domains. One possible conclusion is that propofol interaction sites at β-β and α-β vs. β-α interfaces involve different structures. This is supported by our mutational data on the β3(T266) residue. The β3(T266W) mutation eliminates propofol activation in β3 homomeric receptors. Loss of propofol activation, however, is not reproduced in heteromeric α1β3 receptors containing homologous mutations at all interfaces (α1(I271W)β3(T266W)). Complementary evidence comes from the finding that propofol activation is not eliminated in receptors containing mutations to the β2(M286) residue in α1β2γ2 receptors (Krasowski et al., 2001b). We hypothesize that propofol sites at the β-β and α-β interfaces involve the region between the β3(T266) and β3(F221) residues at the cytoplasmic and extracellular ends, respectively. The propofol site at the β-α interface may involve residues nearer to the cytoplasmic side of the membrane-spanning domains and include the β3(M286), α1(M236), and α1(I239)) residues.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Eaton, Chen, Franks, Evers, and Akk.

Conducted experiments: Eaton, Cao, and Akk.

Contributed new reagents or analytical tools: N/A

Performed data analysis: Eaton, Cao, and Akk.

Wrote or contributed to the writing of the manuscript: Eaton, Franks, Evers, and Akk.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Structures of the $GABA_A$ receptor and the putative propofol binding pocket. (**A**) A top (extracellular) view of the receptor. The five subunits surround a centrally located pore. By convention, the subunits have "+" and "-" sides that are ordered in the fashion shown in the figure. The putative propofol binding site is located at the interface between two neighboring subunits. In β3 homomeric receptors, there are five identical β-β interfaces. Heteromeric α1β3 receptors, likely, have a stoichiometry of two α and three β subunits, and assemble as β-α-β-α-β (top view, counterclockwise). These receptors contain two β-α interfaces, two α-β interfaces, and one β-β interface. (**B**) A side view of two subunits, viewed from the channel lumen in the direction of the arrow in (A). Chain A (yellow) supplies the "-" side of the interface, Chain B (green) supplies the "+" side of the interface. In β3 receptors, both sides of the interface are β subunits. In α1β3 receptors, the yellow chain is a β subunit while the green chain is a β subunit or an α subunit. (**C**) The intersubunit interface at a higher resolution showing the side chains of amino acid residues probed in this study.

Figure 2. Concentration-response properties of human β3 homomeric receptors. The figure shows propofol (**A**) and pentobarbital (**B**) concentration-response curves for wild-type and mutant receptors. The data points show normalized responses (mean ± S.E.M.) from at least four cells at each condition. Current responses were normalized to the response to the highest concentration of agonist used in the experiment. The curves show predictions of Equation 1 generated with the overall mean EC_{50} values from Table 1. In **A**, the maximal fitted response and n_H were 1.05 ± 0.03 and 1.6 ± 0.2 (wild-type), 1.07 ± 0.02 and 1.7 ± 0.1 (H267W), 1.1 ± 0 and 1.6 ± 0.1 (L268W), and 0.99 ± 0.04 and 0.98 ± 0.16 (Y143W). Receptors containing the T266W, Q224W, or F221W mutations did not reliably produce responses in the presence of propofol. In **B**, the maximal fitted response and n_H were 1.03 ± 0.03 and 1.6 ± 0.04 (wild-type),

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1.14 ± 0.03 and 1.8 ± 0.3 (H267W), 1.0 ± 0.03 and 1.8 ± 0.2 (L268W), 1.05 ± 0.04 and 1.1 ± 0.04 (Y143W), 1.03 ± 0.03 and 0.9 ± 0.2 (T266W), and 0.98 ± 0.02 and 2.9 ± 0.04 (Q224W).

Figure 3. Modeling results from studies on human β3 homomeric receptors. (**A**) A simplified allosteric activation model based on (Chang and Weiss, 1999; Colquhoun, 1998; Monod et al., 1965; Rusch et al., 2004). The model describes receptor activation with 5 sites whose affinity to agonist (A) in the closed state is described by K_C and in the open state by K_O. L_O (ratio of C to O) describes opening of unliganded receptors. Opening of fully-liganded receptors is described by L_O multiplied by d (=K_O/K_C) to the fifth power. (**B**) The data points show averaged values for open probability (P_o) of wild-type and mutant β3 receptors activated by propofol. The P_o values were obtained by comparing baseline current level and responses to propofol to a current range spanning from P_o of 0 (determined in the presence of picrotoxin or blocking concentrations of pentobarbital) to P_o of 1 (maximal inward current during or following application of pentobarbital). The curves were generated by fitting Eq. 2 (with n = 5 sites) to the data. Fitting parameters are given in Table 3. (**c**) The data points show averaged values for open probability (P_o) of wild-type and mutant β3 receptors activated by pentobarbital. The P_o values were obtained by comparing baseline current level and responses to pentobarbital to a current range spanning from P_o of 0 to P_o of 1. The curves were generated by fitting Eq. 2 (with n = 4 sites) to the P_o data. Fitting parameters are given in Table 3.

Figure 4. Effects of mutations on receptor activation by propofol and propofol analogs. The graph compares ratios (mean ± S.E.M.) of maximal responses to propofol or propofol analogs to responses to saturating pentobarbital in wild-type and mutant β3 receptors. Propofol was applied at 10-500 μM. Receptors containing the β3(F221W) mutation were not activated by propofol (#). Analogs were applied at 500 μM, except for 2-*tert*-butyl-6-methylphenol that was applied on β3(F221W) and β3(T266W) receptors at 10 μM. Pentobarbital was applied at 300

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μM (wild-type) or 3 mM (mutants). A value of 1 for the calculated parameter means that the compound/pentobarbital current ratio is the same in the mutant and wild-type. The actual compound/pentobarbital current ratios for wild-type and mutant receptors are provided in the text. Statistical analysis (t-test, Excel, Microsoft) was conducted by comparing the calculated parameter value to 1. The data show that the β3(Y143W) mutation does not affect the current ratio for 2-isopropylphenol or 2,6-dimethylphenol while the β3(Q224W) mutation does not affect the current ratio for 2-isopropylphenol or 2-*tert*-butyl-6-methylphenol.

Figure 5. Concentration-response properties of human α1β3 receptors. The figure shows propofol (**A**), pentobarbital (**B**), and GABA (**C**) concentration-response curves for wild-type and mutant receptors. The data points show normalized responses (mean ± S.E.M.) from at least four cells at each condition. Current responses were normalized to the response to the highest concentration of agonist used in analysis. The curves show predictions of Equation 1 generated with the overall mean EC_{50} values from Table 3. In cases where no clear saturation was observed but higher drug concentrations produced block, Y_{max} was constrained to the value of the highest current response. In **A**, the maximal fitted response and n_H were 1.07 ± 0.01 and 1.7 ± 0.3 (α1β3), 1.0 (constrained) and 4.3 ± 0.7 (α1β3(T266W)), 1 and 2.0 ± 0.1 (α1(I271W)β3), and 0.98 ± 0.02 and 2.9 ± 0.4 (α1(I271W)β3(T266W)). In **B**, the maximal fitted response and n_H were 1 and 2.1 ± 0.3 (α1β3), 1.07 ± 0.03 and 0.9 ± 0.06 (α1β3(T266W)), 1.02 ± 0.01 and 2.4 ± 0.1 (α1(I271W)β3), and 1 and 2.9 ± 0.6 (α1(I271W)β3(T266W)). In **C**, the maximal fitted response and n_H were 1.04 ± 0.01 and 1.5 ± 0.1 (α1β3), 1.07 ± 0.06 and 0.8 ± 0.1 (α1β3(T266W)), 1.0 and 2.0 ± 0.1 (α1(I271W)β3), and 1.04 ± 0.03 and 1.3 ± 0.2 (α1(I271W)β3(T266W)).

Table 1. Propofol and pentobarbital concentration-response data for β3 homomeric receptors.

Receptor	Propofol EC_{50} (μM)	Pentobarbital EC_{50} (μM)	$I_{propofol}/I_{pentobarbital}$ (%)	$P_{o,spont}$
β3 wild-type	9 ± 1	59 ± 7	53 ± 3	0.13 ± 0.02
β3(H267W)	10 ± 1	67 ± 20	27 ± 1	0.07 ± 0.02
β3(L268W)	15 ± 2	272 ± 40	14 ± 1	0.01 ± 0.001
β3(Q224W)	N/A	666 ± 21	<3	0
β3(F221W)	N/A	523 ± 45	0	0.62 ± 0.04
β3(Y143W)	22 ± 6	82 ± 24	13 ± 4	0.21 ± 0.02
β3(T266W)	N/A	389 ± 81	<4	0.34 ± 0.08

The concentration-response data from each cell were fitted with Equation 1 (Methods). The table shows propofol and pentobarbital EC_{50} values (mean ± S.E.M.) from at least 4 cells under each condition. Propofol activation was minimal in receptors containing the Q224W, F221W, or T266W mutations; for these receptors concentration-response relationship was not determined. $I_{propofol}/I_{pentobarbital}$ was calculated by measuring responses to saturating propofol and saturating pentobarbital in the same cell. Cells expressing β3(F221W) did not respond with inward current to application of propofol. Open probability of unliganded receptors ($P_{o,spont}$) was calculated assuming that P_o reached 0 in the presence of 10-100 μM picrotoxin and 1 in the presence of saturating pentobarbital. The β3(F221W) exhibited greater block during the initial application of 3 mM pentobarbital than in the presence of picrotoxin. Accordingly, we compared block by pentobarbital to the maximal rebound response to pentobarbital to calculate $P_{o,spont}$ in β3(F221W).

Table 2. Fitted propofol and pentobarbital binding and gating parameters for allosteric activation model.

Receptor	K_C, propofol (μM)	*d*, propofol	K_C, pentobarbital (μM)	*d*, pentobarbital
β3 wild-type	8.8	0.64	1022	0.07
β3(H267W)	7.1	0.67	3482	0.02
β3(L268W)	6.5	0.55	359	0.16
β3(Q224W)	N/A	N/A	2401	0.05
β3(F221W)	N/A	N/A	>40 mM	0.06
β3(Y143W)	14.4	0.91	118	0.34
β3(T266W)	N/A	N/A	1503	0.34

Pooled P_o data from at least four cells were fitted with Equation 2 (Methods). L_o was calculated as $(1\text{-}P_{o,spont})/P_{o,spont}$, equaling 6.7 (for wild-type), 13.3 (H267W), 99 (L268W), 0.7 (F221W), 3.8 (Y143W), or 1.9 (T266W). For Q224W that showed no spontaneous activity, we used an arbitrary L_o value of 1000. K_C is the equilibrium dissociation constant for closed receptors. Parameter *d* equals the ratio of open receptor and closed receptor dissociation constants (K_O/K_C), and is a measure of gating efficacy (large values denote poor

efficacy). The goodness of the fit showed minimal changes when the number of propofol or pentobarbital binding sites varied between 3 and 5. The parameters in the table are from fits with 5 propofol and 4 pentobarbital binding sites.

Table 3. Propofol and pentobarbital concentration-response data for α1β3 receptors.

Receptor	Propofol EC_{50} (μM)	Pentobarbital EC_{50} (μM)	GABA EC_{50} (μM)	$I_{propofol}/I_{GABA}$ (%)	$I_{pentobarbital}/I_{GABA}$ (%)
α1β3 wild-type	13 ± 2	87 ± 13	1.4 ± 0.1	37 ± 5	21 ± 3
α1(I271W)β3	5 ± 1	68 ± 5	0.6 ± 0.1	89 ± 3	91 ± 1
α1(I271W)β3(T266W)	9 ± 1	113 ± 30	3.3 ± 0.7	76 ± 3	87 ± 3
α1β3(T266W)	4 ± 1	166 ± 42	0.2 ± 0.04	74 ± 1	93 ± 4

The concentration-response data from each cell were fitted with Equation 1 (Methods). The table shows propofol, pentobarbital, and GABA EC_{50} values (mean ± S.E.M.) from at least 4 cells under each condition. Relative currents were estimated by comparing responses to saturating propofol, pentobarbital, and GABA in the same cell. Saturating GABA produces responses with the P_o of near 1, because co-application of 1 μM alphaxalone did not modify peak responses to saturating GABA (not shown).

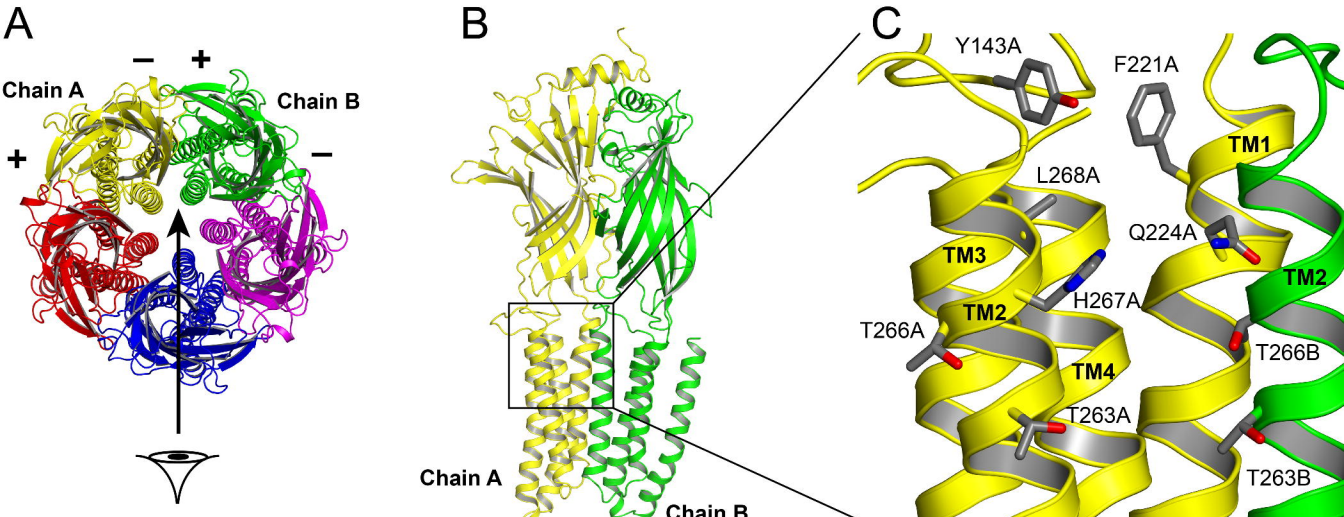

A
Chain A
Chain B
B
Chain A
Chain B
C
Y143A
F221A
TM1
L268A
Q224A
TM3
TM2
H267A
TM2
T266A
T266B
TM4
T263A
T263B

Figure 2

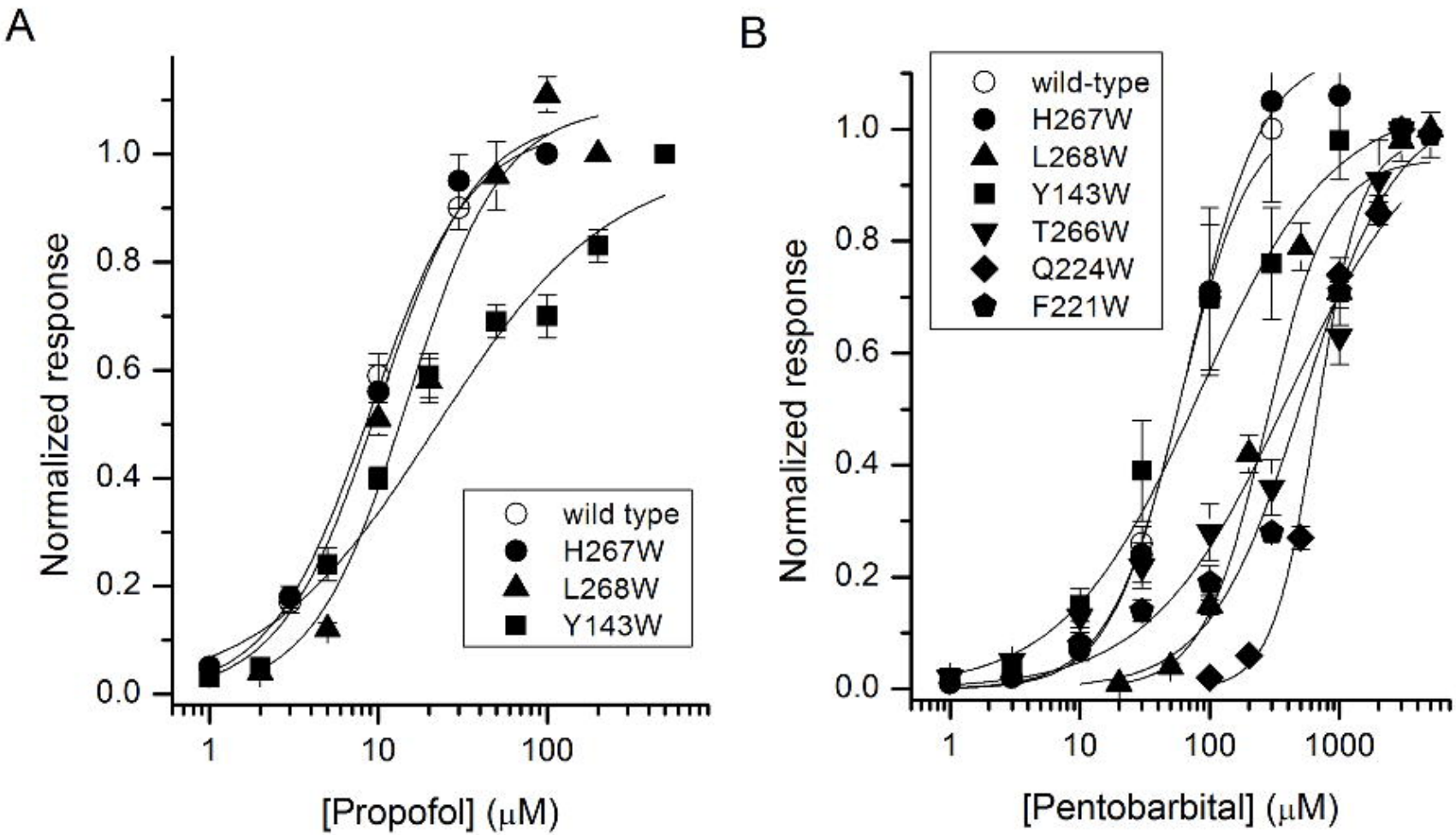

A
Normalized response
1.0
0.8
0.6
0.4
0.2
0.0
1
10
100
[Propofol] (μM)
wild type
H267W
L268W
Y143W
B
Normalized response
1.0
0.8
0.6
0.4
0.2
0.0
1
10
100
1000
[Pentobarbital] (μM)
wild-type
H267W
L268W
Y143W
T266W
Q224W
F221W

Figure 3

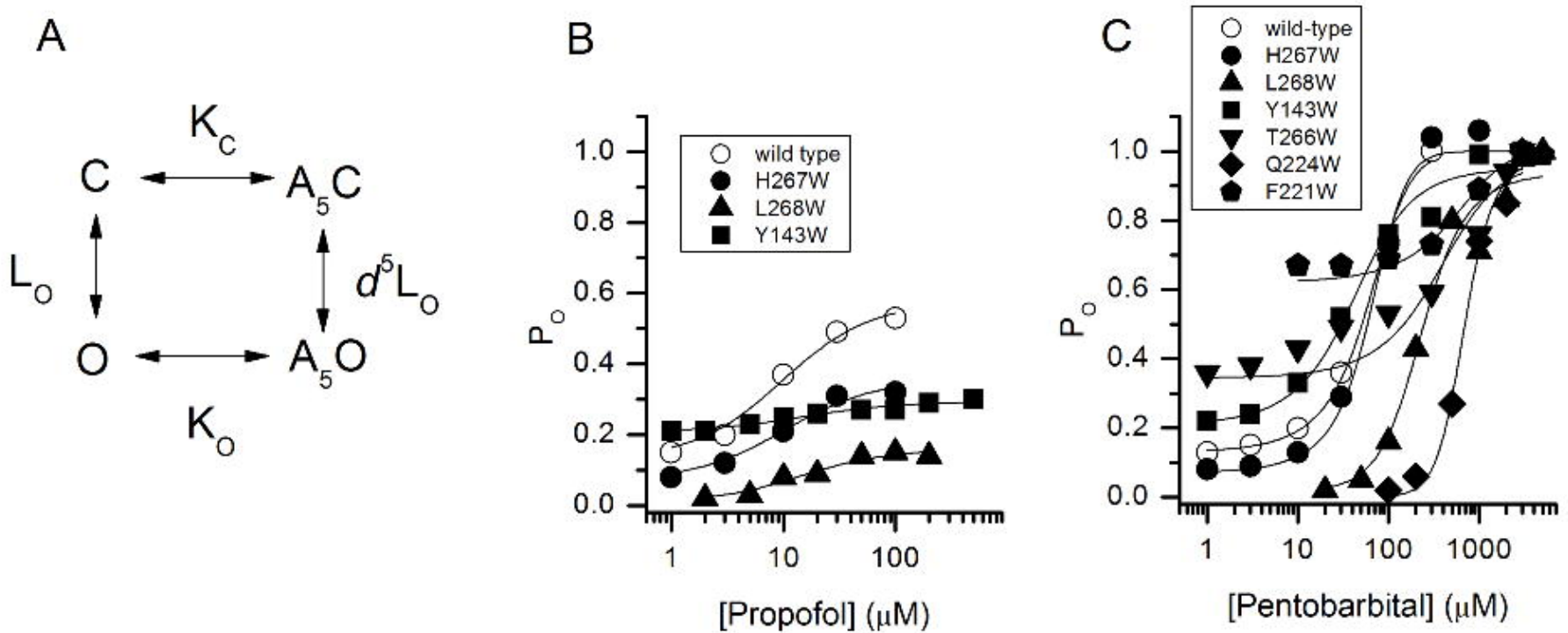
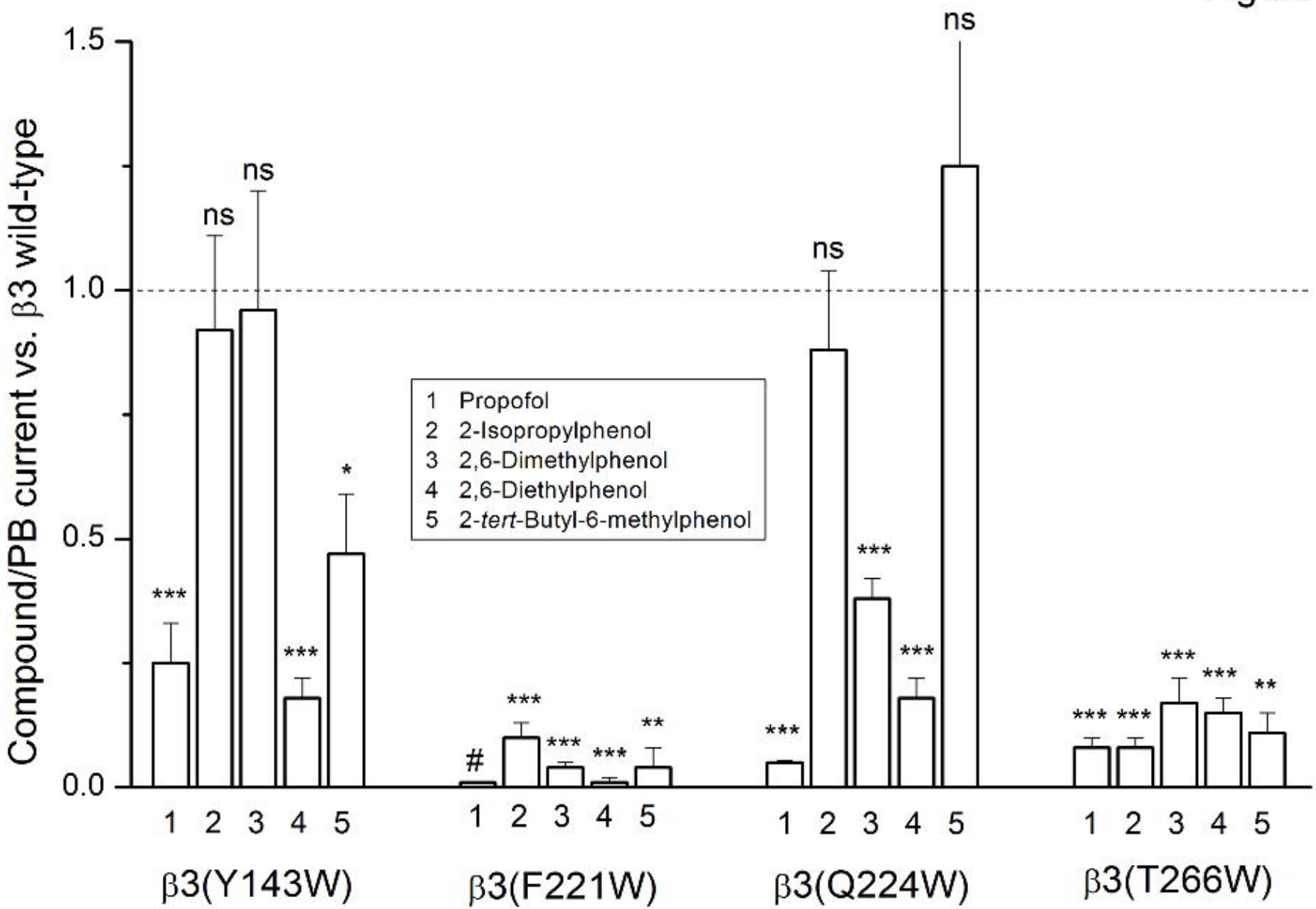

A
K_C
C
A_5C
L_O
d^5L_O
O
A_5O
K_O
B
wild type
H267W
L268W
Y143W
P_O
1.0
0.8
0.6
0.4
0.2
0.0
1
10
100
[Propofol] (μM)
C
wild-type
H267W
L268W
Y143W
T266W
Q224W
F221W
P_O
1.0
0.8
0.6
0.4
0.2
0.0
1
10
100
1000
[Pentobarbital] (μM)

Figure 4


Compound/PB current vs. β3 wild-type
1.5
1.0
0.5
0.0
1 Propofol
2 2-Isopropylphenol
3 2,6-Dimethylphenol
4 2,6-Diethylphenol
5 2-tert-Butyl-6-methylphenol
ns
ns
ns
ns
*
***
***
#
***
***
***
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***
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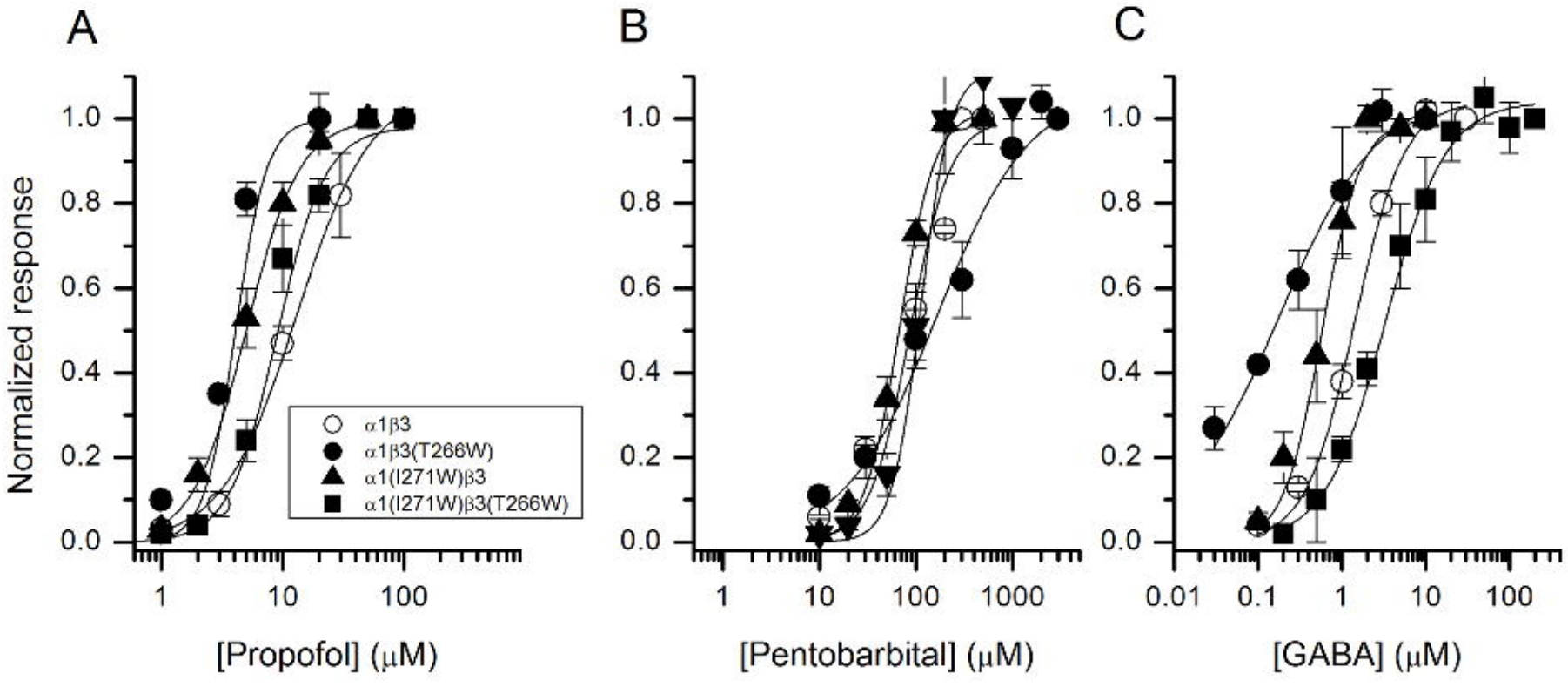
***
***
***
***
**
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
β3(Y143W)
β3(F221W)
β3(Q224W)
β3(T266W)

Figure 5


A
B
C
Normalized response
1.0
0.8
0.6
0.4
0.2
0.0
α1β3
α1β3(T266W)
α1(I271W)β3
α1(I271W)β3(T266W)
1
10
100
[Propofol] (μM)
1000
[Pentobarbital] (μM)
0.01
0.1
[GABA] (μM)