

1 Abstract

GABA(A), a pentameric ligand gated ion channel is critical for regulating neuronal excitability. These inhibitory receptors, gated by γ -amino butyric acid (GABA), can be potentiated and also directly activated by intravenous and inhalational anesthetics. Although this receptor is a widely-studied target for general anesthetics, the mechanism of receptor modulation remains unclear. These receptors are predominantly found in $2\alpha: 2\beta: 1\gamma$ stoichiometry, with four unique inter-subunit interfaces. Here we use thermodynamically rigorous free energy perturbation (AFEP) techniques and Molecular Dynamics simulations to rank the different intersubunit sites by affinity. AFEP calculations predicted selective propofol binding to interfacial sites, with higher affinities for $\alpha_\beta - \beta_\alpha$ and $\beta_\gamma - \alpha_\beta$, $\gamma - \beta_\gamma$, and is equivalent to propofol EC50. Propofol is predicted to have 10-fold lower affinity at the other identical site, $\beta_\alpha - \alpha_\gamma$. The simulations revealed the key interactions leading to propofol selective binding within GABA(A) receptor subunit interfaces, with stable hydrogen bonds observed between propofol and β subunit at $\alpha_\beta - \beta_\alpha$ and $\gamma - \beta_\gamma$ sites. Varying number of water and lipid molecules flooding the site along with multiple hydrogen bonding partners, causes some differences in affinities among the 5 intersubunit sites. Propofol competes with water and lipid molecules for hydrogen bonding in the more amphiphilic and less tight binding site, $\alpha_\beta - \gamma$ due to the lack of bulky residues at $15'M3-\alpha$ and $15'M1-\gamma$ thus resulting in a lower affinity.

Weaker affinities were measured for sevoflurane, consistent with its greater EC50. 'Flooding' molecular dynamics simulations identified stable binding modes in the accessible $\gamma - \beta_\gamma$, $\alpha_\beta - \beta_\alpha$ and $\beta_\alpha - \alpha_\gamma$ sites. Flooding simulation also reveals sites that show prefer lipid binding over Sevoflurane and site with multiple occupancy. AFEP calculations predicted Sevoflurane to have affinity equivalent to its EC50 in all intersubunit sites and show no specificity to any particular site. Flooding simulation also reveals a site with multiple occupancy.

2 Introduction

General anesthetics has been shown to have act at multiple targets, ligand-gated ion channels (3–5), and in particular, GABA(A) receptors, have been identified as primary targets for widely-used general anesthetics like propofol and sevoflurane.(6, 7).

The γ -amino butyric acid type A (GABA_AR) receptor is an ionotropic receptor critical for inhibitory signaling in the central nervous system. GABA_AR exists as heteropentamers, predominantly in the $2\alpha: 2\beta: 1\gamma$ stoichiometry (8–10). Each subunit consists of 4 helices (M1-M4) in the transmembrane domain, with M2 lining the pore and M4 facing the lipid membrane. Numerous molecules with sedative, anxiolytic, and anesthetic properties are positive modulators or agonists of the GABA(A) receptor, including neurosteroids (11), benzodiazepines (12, 13) and inhalational anesthetics such as sevoflurane (14–16) and intravenous general anesthetics (7, 17)like propofol (18).

Propofol has been a predominantly used general anesthetic since its discovery in 1980. Propofol has been shown to potentiate GABA_AR (19) and even directly activate the channel at higher concentrations (20, 21). With the lack of anesthetic bound crystal structure of GABA_AR, identifying binding sites has been mainly through indirect means of mutagenesis and photolabelling. While certain studies have suggested sites involving α or γ subunit, extensive site-directed mutagenesis and photo-labelling indicates a compulsory presence of β subunits in the binding sites (22–27). With the surge of efficient photo-analogs developed in the recent times for multiple anesthetics that target GABA_AR, studies have been able to find relative affinities for specific binding sites (28). This study further indicates the presence of atleast 4 distinct binding sites ($\beta_+ - \alpha_-$; $\alpha_+/\gamma_+ - \beta_-$) for propofol with varying affinities.

Among the inhaled anesthetics, isoflurane was the first anesthetic shown to enhance GABA induced currents(14), following which most volatile anesthetics have been shown to positively modulate GABA_AR at a concentration($\approx 300\mu\text{M}$) much lower than that of intravenous anesthetics(29). Mutagenesis and electrophysiology studies have identified α subunit to be more significant for potentiation by sevoflurane than β subunit(30). Mutagenesis studies usually suffers the disadvantage of misinterpreting the results from allosteric conformational change and developing a photoaffinity analogue closely resembling the parent compound has been very challenging. Computational approaches can complement the experimental data, and can be useful in analyzing protein-ligand interactions. Although docking has been used to approximate the location of the binding site, the docking algorithm does not account for desolvation, rotational and translational entropy of the bound ligand and the protein dynamics(31). In contrast, MD simulations involve simulating the anesthetic-bound receptor along with the lipid membrane and explicit water, allowing the ligand to explore the binding site. A recent study involved using a novel photoaffinity analog of Propofol, showed selectivity to sites, $\beta_+ - \alpha_-$ or $\alpha_+ - \beta_-$ and this was further substantiated using MD simulations to identify key interactions mediating the binding of the ligand and obtain KD values explaining the affinity differences between α/β sites and sites involving γ subunits.

3 Methods

Simulations: The manuscript contains data from four systems; $\alpha_1\beta_3\gamma_2$ apo receptor ; These simulations, run for 120ns, were used to understand the nature of the binding sites in the absence of the ligands. These simulations served as a control for the measuring the convergence of the Free energy perturbation simulations.

Anesthetic bound $\alpha_1\beta_3\gamma_2$; Two separate system were setup; *Propofol-* $\alpha_1\beta_3\gamma_2$ and *Sevoflurane-* $\alpha_1\beta_3\gamma_2$, with propofol and sevoflurane docked to the transmembrane domain of the receptor, respectively, using the Autodock software(32). The search space for docking were chosen based on the binding site residues identified in previous experimental studies and as detailed in the book chapter(31). *Propofol-* $\alpha_1\beta_3\gamma_2$ system were run for \approx 600ns and *Sevoflurane-* $\alpha_1\beta_3\gamma_2$ system were run for \approx 150ns.

Sevoflurane flooded $\alpha_1\beta_3\gamma_2$ system; Sevoflurane was inserted randomly into the water surrounding the protein, with an sevoflurane-to-lipid ratio of about 1?3. The simulation was run for \approx 1.7 μ s.

GABA_AR receptor, in this study, is modelled based on the GluCl crystal structure(4RHW) with the ivermectin, a positive modulator, bound to the TMD (M2-15') of the channel (33). This confirms the presence of 5 distinct binding clefts at inter-subunit sites in the TMD region. The GABA_AR receptor is arranged clockwise with two α 1, two β 3, and one γ 2 subunit arranged $\beta\alpha\beta\alpha\gamma$ counterclockwise. This creates 5 intersubunit sites, $\beta_\gamma - \alpha_\beta$, $\alpha_\beta - \beta_\alpha$, $\beta_\alpha - \alpha_\gamma$, $\alpha_\gamma - \gamma$, $\gamma - \beta_\gamma$ (Figure 1 B).

System setup: The systems were prepared as in Ref(34), by embedding the protein in a lipid bilayer composed of phosphatidylcholine, built using CHARMM Membrane builder, with the final system containing 268 POPC molecules. The systems were solvated using the SOLVATE plugin in VMD(35) and neutralizing ions were added to bring the system to a 0.15M salt concentration using the AUTOIONIZE plugin. The final system contained about 160,000 atoms.

All simulations used the CHARMM22-CMAP(36) force field with torsional corrections for proteins. The CHARMM36 model(37, 38) was used for phospholipids, ions, water and cholesterol molecules. Energy minimization and MD simulations were conducted using the NAMD2.11 package(39). All simulations employed periodic boundary conditions, long-ranged electrostatics were handled with smooth particle mesh Ewald method, and a cutoff of 1.2 nm was used for Lennard-Jones potentials with a switching function starting at 1.0 nm. All simulations were run in the NPT ensemble with weak coupling to Langevin thermostat and a barostat at a respective 300 K/315 K and 1 atm. All bonds to the hydrogen atoms were constrained using the SHAKE/RATTLE algorithm. A multiple time-step rRESPA method was used, and controlled with a high frequency time-step of 2fs and low frequency time-step of 4fs.

All standard MD simulations with bound anesthetic were energy minimized for 10000 steps. Harmonic restraints of 0.5 kcal/mol/ \AA were used on the backbone of the protein to restrain the system in a open conformation.

Free energy perturbation simulations were performed on *Propofol-* $\alpha_1\beta_3\gamma_2$ and *Sevoflurane-* $\alpha_1\beta_3\gamma_2$ systems, with starting configurations obtained from running standard MD simulations. The force constants for binding site restraints were on the order of 5 kcal/mol/ \AA . The movement of ligand was confined to 5 \AA of the binding site by using a spherical flat-bottom restraint. The decoupling of the ligand was performed over a series of windows. Perturbation parameter λ was sampled with a step size equal to 0.025 between 0; λ ; 0.1 and 0.9; λ ; 1, and 0.05 otherwise. Each step in 1 started with a 4 ps equilibration period followed by a 5 ns run for data collection. Free energy of binding was calculated as in Ref (?)

Images: All the images were created using VMD(35). The VOLMAP plug-in was used to create an image depicting the average density of sevoflurane near the TM of the channel. Tcl and Python scripts were used to depict the correlation between binding affinity and water/lipid displacement from binding sites.

4 Results

4.0.1 Persistent interactions observed between anesthetics and residues from photolabeling

Various residues in the intersubunit sites of GABA_AR have been identified, experimentally, as possible binding sites for propofol (27)and sevoflurane (43), as shown in Figure 1 A and Table 1. Propofol bound GABA_AR simulations revealed how propofol interacts these residues ((Figure 2). Table 1 further indicates the average distance of the residues from the bound propofol in the course of the simulation. Sites, $\gamma - \beta_\gamma$, $\beta_\gamma - \alpha_\beta$, $\alpha_\beta - \beta_\alpha$, $\beta_\alpha - \alpha_\gamma$ have two photolabelled residues, in addition to a residue identified though mutagenesis in site $\beta_\alpha - \alpha_\gamma$. No residue has been identified in the $\alpha_\gamma - \gamma$ site. Of all the experimentally identified residues, β M227, β M286, α M236, β N265, are in close proximity to the bound propofol (Table 1). β N265 is the only residue that shows possibility of hydrogen bond formation with propofol (Figure 4B,D)

All of the photolabelled residues for sevoflurane , with AziSEVO(43), are part of the lower TM region of the channel. Flooding simulation with sevoflurane revealed the proximity of the photolabelled residues with sevoflurane. Most of the photolabelled residues face into pore , while some residues face into the lipid membrane (Figure 3 E). While majority of the sevoflurane molecules, flooding the system, bound to the upper TM of the channel, some of the molecules also bound to periphery of the channel (Figure 3E). As shown in Table 1, β W241, which faces the lipids, comes in close proximity to sevoflurane molecules, flooding the peripheral of the protein.

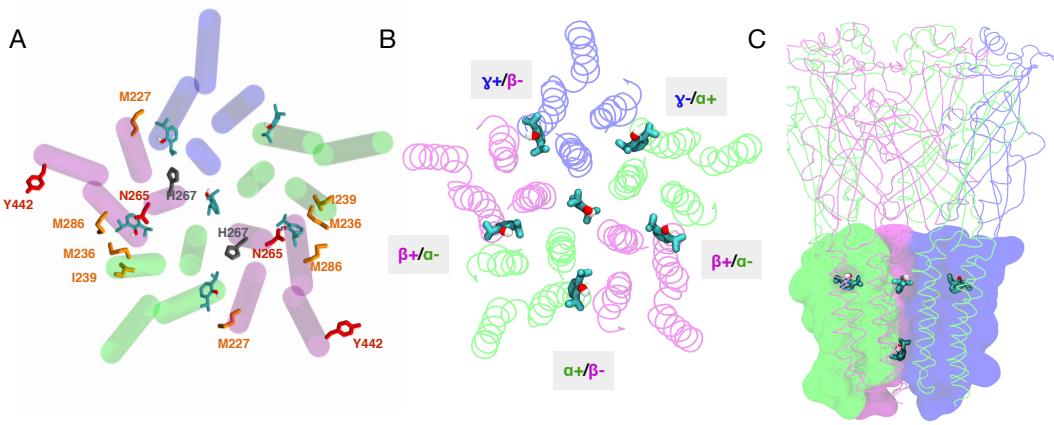


Figure 1: View of the TMD of GABA_AR from the ECD GABA_AR is colored by subunit (A) Propofol binding site residues identified through photolabelling using AziPM are shown in orange; o-PD are shown in Gray and residues identified through mutagenesis are shown in red; (B) View of the TMD of GABA_AR from the ECD; Starting conformation of propofol in the intersubunit sites are shown in licorice form; (C) Cross-section view of the channel showing the starting conformation of PFL bound to TMD of GABA_AR.

Table 1: Experimentally identified binding site residues of propofol and sevoflurane(?) in GABA_AR. The first column indicates the location of the residue in the receptor; second column indicates the subunit, helix, resname and resid of the residue; third column indicates average distance over 600ns, between center of mass of binding site residue and the nearest propofol; distances from last frame of flooding simulations, between center of mass of binding site residue and the nearest sevoflurane.

Propofol binding site	Helix/Residue	distance(Å)
$\gamma - \beta_\gamma; \alpha_\beta - \beta_\alpha$	β M1 M227 (40)	7;7
	β M2 H267 (41)	10;12
$\beta_\gamma - \alpha_\beta; \beta_\alpha - \alpha_\gamma$	β M2 N265 (42)	5;6
	β M3 M286 (40)	6;7
	α M1 M236 (40)	7;7
	α M1 I239 (40)	13;12
Sevoflurane binding site	Helix/Residue	distance(Å)
intrasubunit	α M1 C234	12;9
	α M2 R255	13;14
	γ M2 G269	15
	α M1 S241	13;10
pore facing	α M2 P253	19;17
	α M2 V257	14;15
	α M2 T261	10;11
	β M2 A248	17;20
Lipid facing	β M1 W241	4;9
$\gamma - \beta_\gamma; \alpha_\beta - \beta_\alpha$	α M2 V260	13;13
	β M2 A249	14;20
$\beta_\gamma - \alpha_\beta; \beta_\alpha - \alpha_\gamma$	α M2 T265	9;10
	β M2 I255	12;12
$\alpha_\gamma - \gamma$	γ M2 L268	12

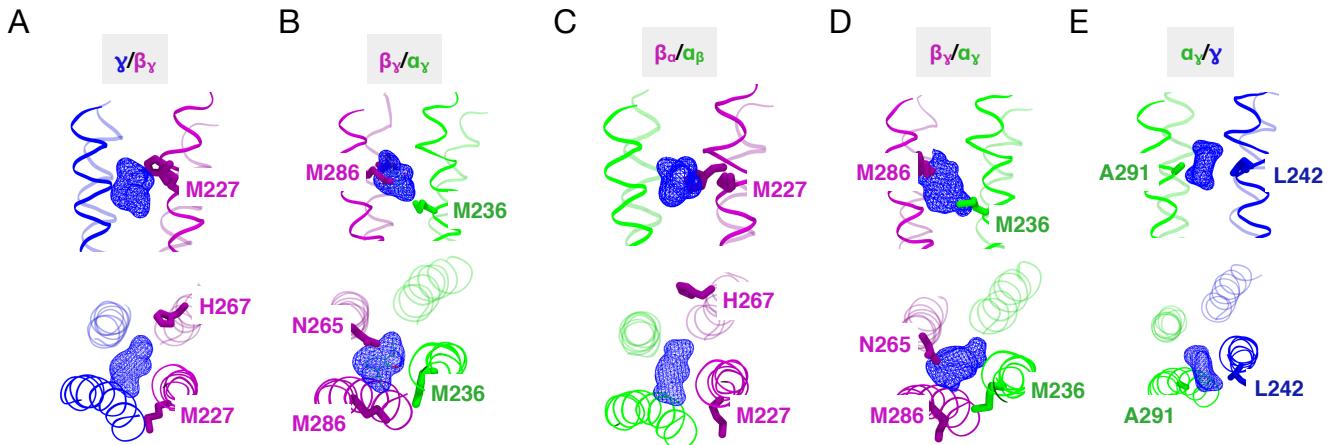


Figure 2: Trajectory of propofol at subunit interface. Individual subunit interface, with view from ECD(top) and view along TMD(below); Licorice residues colored by subunit are the residues identified through previous experimental studies; Blue dots represent the center of mass of propofol throughout the simulation(A) $\gamma - \beta_\gamma$, (B) $\beta_\gamma - \alpha_\beta$, (C) $\alpha_\beta - \beta_\alpha$, (D) $\beta_\alpha - \alpha_\gamma$, (E) $\alpha_\gamma - \gamma$; (E) No residues have been reported in this site; Residues in licorice form are residues homologous to other sites.

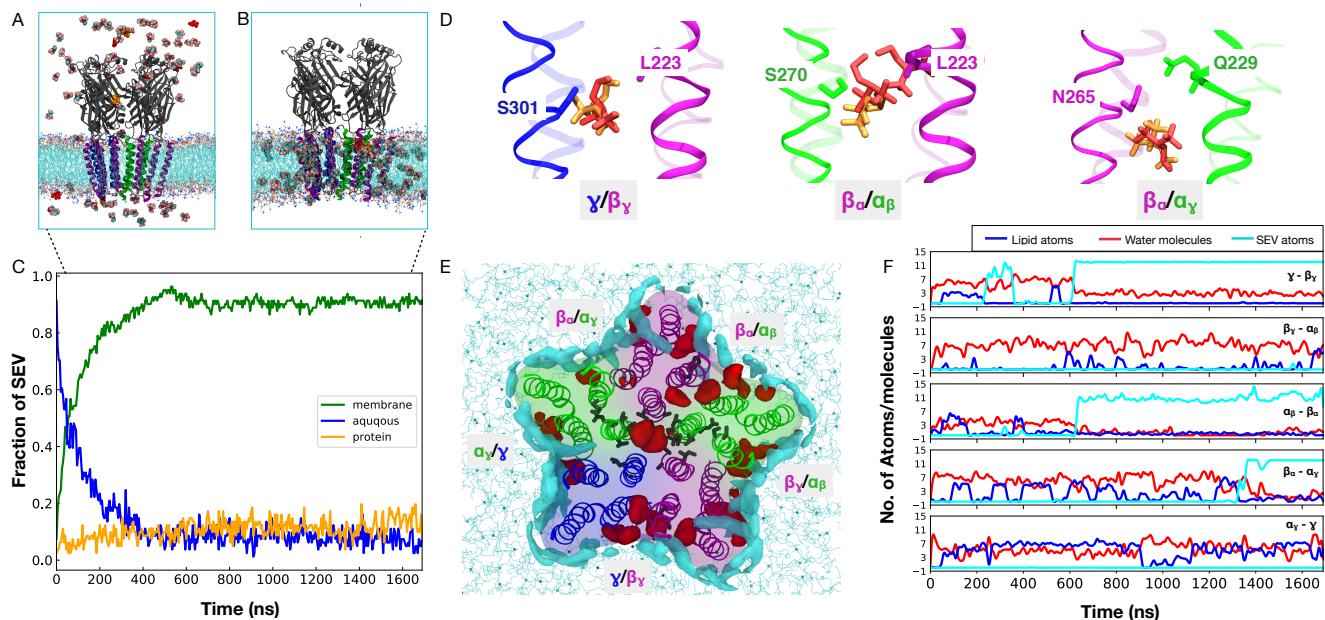


Figure 3: Flooding simulation of GABA_AR with sevoflurane. (A) GABA_AR system flooded with sevoflurane in the water box. (B) GABA_AR system after the sevoflurane partitions into the lipid membrane; (C) fraction of sevoflurane molecules in each phase; (D) Different intersubunit sites viewed from the TMD, depicting the binding sites and orientation of sevoflurane identified through flooding simulation(red) and standard MD simulation(orange); (E) View of the TMD of GABA_AR from the ECD at the final frame of the flooding simulation, displaying the average density of sevoflurane molecules and lipids bound to the TMD of the channel; (F) Number of water(red) molecules, lipid(blue) and Sevoflurane(cyan) atoms that enter intersubunit cavity in the course of the simulation.

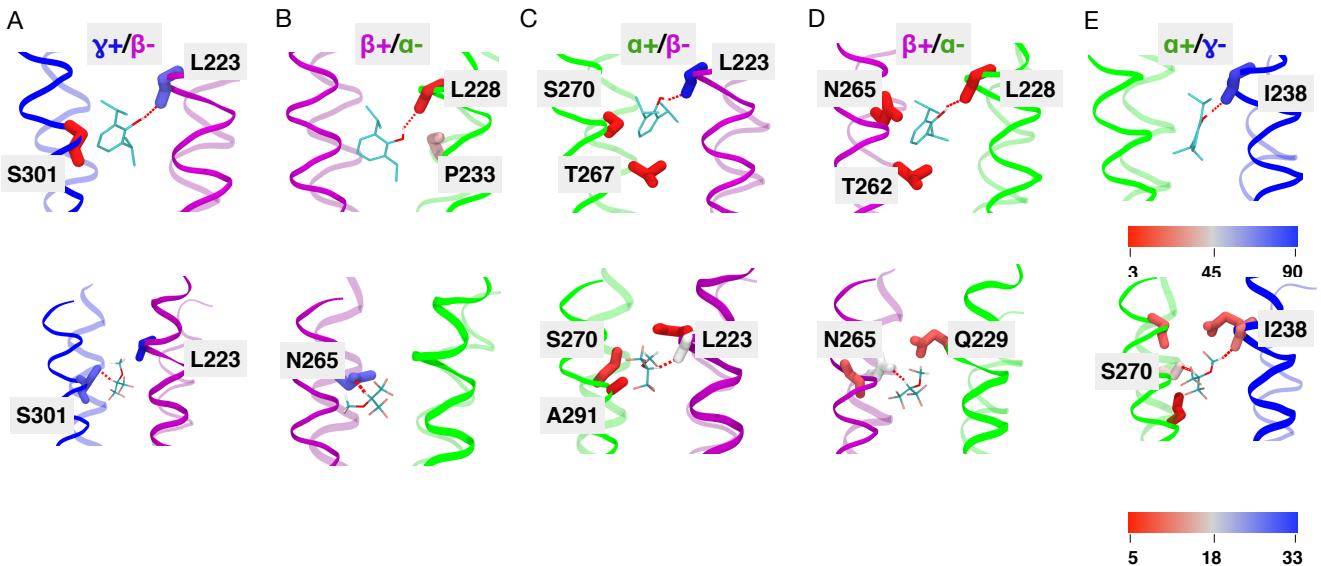


Figure 4: Protein-anesthetic interactions in intersubunit sites. (A) Percentage of H-bonds between protein or water and (Top) Propofol and (Bottom) Sevoflurane. The protein residues that H-bond with PFL(top) and SEV(Bottom) are shown in licorice and colored by the percentage of the hydrogen bonds formed in the course of the simulations, with red denoting residues that form least number of Hydrogen bonds with the ligand and blue denoting the residues forming the highest number of hydrogen bonds.

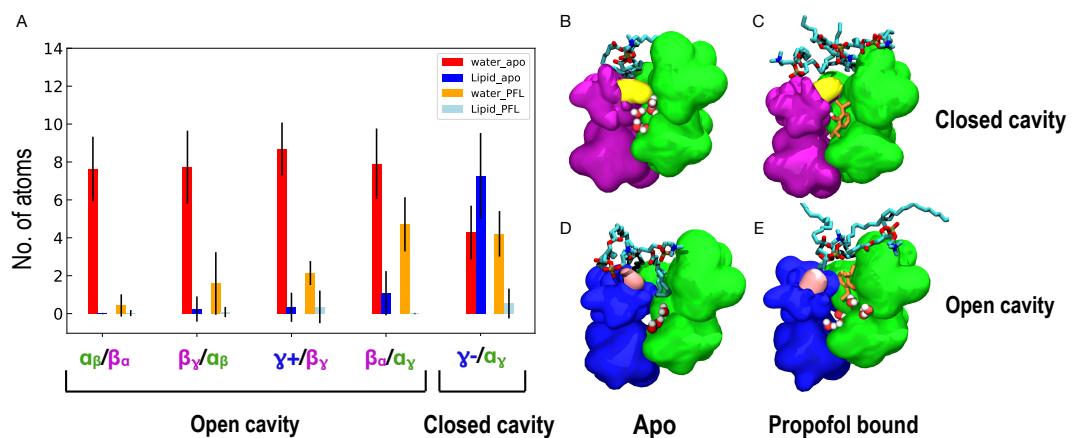


Figure 5: Open and closed cavities (A) Plot depicting the number of water or lipid molecules in Apo and propofol bound receptor. (B,C) and (D,E) shows a snap-shot of the open and closed cavity in Apo (B,D) and propofol-bound receptor(C,E), viewed from the ECD. The methionine residue forming the closed cavity is colored in yellow; the isoleucine residue forming the open cavity is colored in pink.

4.0.2 Flooding with sevoflurane suggests multiple occupancy for some sites, as well as exchange with lipid.

As shown in Figure 3(E,F), we observe sevoflurane flooded in the system to occupy three of the five intersubunit sites, $\gamma - \beta_\gamma$, $\alpha_\beta - \beta_\alpha$, $\beta_\alpha - \alpha_\gamma$. Specifically in site, $\alpha_\beta - \beta_\alpha$, we see two sevoflurane bind the site (3(E)), with one of the sevoflurane molecule, entering from the pore, while the other entering from the lipid membrane. Sevoflurane being a small-molecule anesthetic, with higher solubility in water is well suited for a flooding simulation. In ≈ 300 ns we see that almost all of the sevoflurane molecules partition into the lipid membrane, leaving the aqueous environment as shown in Figure (3 A,B, C). Following this we observed sevoflurane to bind inter-, intra-subunit and pore sites (Figure 3 E). All the intersubunit sites

Table 2: Binding affinities of propofol and sevoflurane bound to five GABA_{AR} receptor interfacial sites, calculated using AFEP

Binding site	Propofol - K_D (μM)	Sevoflurane - K_D (μM)
$\alpha_\beta - \beta_\alpha$	0.2 (0.1)	14 ± 6
$\beta_\gamma - \alpha_\beta$	0.2	6 ± 4
$\gamma - \beta_\gamma$	0.6 (200)	215 ± 54
$\beta_\alpha - \alpha_\gamma$	20 (2)	145 ± 103
$\alpha_\gamma - \gamma$	60 (27)	116 ± 68

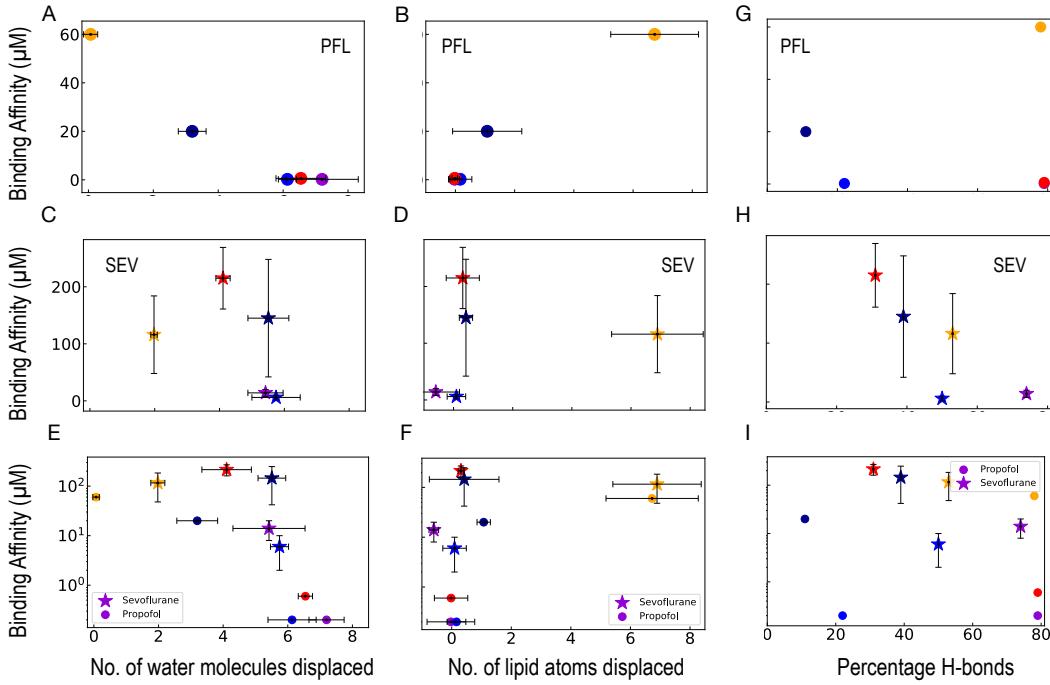


Figure 6: **Water, lipid interactions at intersubunit sites.** (A),(B) Correlation between binding site affinity and average no. of water or lipid atoms displaced in the respective sites in propofol bound receptor system. (C), (D) Correlation between binding site affinity and average no. of water or lipid atoms displaced in the respective sites in sevoflurane bound receptor system.(E),(F) Comparison of correlation between, displacement of water(E) and lipids(F) by sevoflurane/propofol, and their respective binding affinity.(G),(H) Correlation between binding site affinity and hydrogen bonding between propofol/sevoflurane and protein.

identified were in the upper TMD , closer to ECD, similar to sites identified through standard MD simulations . The different inter-subunit sites were occupied by water , lipids or sevoflurane molecules as described in the Figure (3 F). As evident in Figure (3 F) , sevoflurane temporarily occupies $\gamma - \beta_\gamma$ at $\approx 200\text{ns}$, for $\approx 150\text{ns}$ before re-entering the site at $\approx 600\text{ns}$. We see $\alpha_\beta - \beta_\alpha$ site gets occupied at $\approx 600\text{ns}$ as well. While the $\gamma - \beta_\gamma$ site appears to have about 5-8 molecules of water until occupied by sevoflurane , the $\alpha_\beta - \beta_\alpha$ has only about 2-5 molecules of water indicating the more hydrophobic nature of the site. Subsequently, another sevoflurane molecule enters the $\alpha_\beta - \beta_\alpha$ site at $\approx 1\mu\text{s}$ thus revealing a possibility of multiple occupancy at this site. The $\beta_\alpha - \alpha_\gamma$ was the last to get filled in the course of the simulation at $\approx 1.3\mu\text{s}$. Sites $\beta_\gamma - \alpha_\beta$ and $\alpha_\gamma - \gamma$ remained unoccupied in the course of $2\mu\text{s}$ simulation and is instead occupied by lipids and water molecules thus prohibiting sevoflurane from binding(Figure 3 E,F) . All the Sevoflurane molecules bind the inter-subunit sites by entering the lipid membrane except the site $\alpha_+ - \beta_-$ in proximity to the pore, which is bound by a sevoflurane initially present in the pore. This pathway gives us a clear indication of how a ligand entering the pore could end up occupying an inter-subunit site, instead of blocking the pore (44).

4.0.3 Spontaneous binding is observed for intrasubunit, pore, sites from flooding.

As shown in Figure (3 E) β subunit is the only subunit that favored intra-subunit sevoflurane binding , at a height similar to that of the inter-subunit sites. While the site between the M1 and M4 helix is occupied by sevoflurane, a lipid tail is seen to penetrate the subunit between the M3 and M4 helix. This interactions occur at identical spots in both the β subunits. We see three Sevoflurane molecules enter the pore one after the other through the ECD. While two sevoflurane molecules remain very mobile within the upper-TMD of the channel, a third Sevoflurane entering the site leads to it being forced to enter a intersubunit site. In the course of the simulation, one of the sevoflurane molecule enters the inter-subunit site $\alpha_+ - \beta_-$ site from the pore thus revealing another binding site at this cavity.

4.0.4 Propofol but not sevoflurane persistently hydrogen-bonds with backbone.

As shown in Figure 4 (A,B) , Propofol forms Hydrogen bonds with the backbone carbonyl oxygen in each of the interface, more persistently ($\approx 80\text{-}90\%$) in sites with Propofol facing β .and γ subunit than α .. In comparison, Sevoflurane, a less potent anesthetic, forms hydrogen bonds transiently ($\approx 30\%$) than propofol, with having strongest interaction at $\gamma - \beta_\gamma \beta_\gamma - \alpha_\beta$ (Figure 4 (C,D) . Due to the presence of a conserved proline residue at the 13° position on all M1 transmembrane helix, a break in the helix is formed at 16° position, thus causing the carbonyl oxygen to be available for hydrogen-bonding with the ligand. This behavior is also observed in crystal structures of GluCL and GABA β_3 homopentamer.

As illustrated in Figure 4 (B) at the interfaces, $\gamma - \beta_\gamma$, $\alpha_\beta - \beta_\alpha$ and $\alpha_\gamma - \gamma$, the propofol interacts solely with the backbone carbonyl oxygen of β L223, γ I238 respectively. In the two identical $\beta_\alpha - \alpha_\gamma$ and $\beta_\gamma - \alpha_\beta$ sites, propofol behaves differently, with forming highly transient hydrogen bonds with α L228 in $\beta_\gamma - \alpha_\beta$ and weak hydrogen bonds with multiple residue such as , β N265, β N262, and α L228 in $\beta_\alpha - \alpha_\gamma$.

Figure 4(D), sevoflurane forms most consistent hydrogen bond at the $\gamma - \beta_\gamma$ site, with residues β L223 and γ 301. At the $\alpha_\gamma - \gamma$, sevoflurane shows strong interaction with α S270 and weak interactions with the *gamma* interface, Q238, I239. In the $\beta_\gamma - \alpha_\beta$ site, sevoflurane interacts with the residue homologous to α S270, β N265, more consistently than $\beta_\alpha - \alpha_\gamma$. Sevoflurane form weakest interactions at the $\alpha_\beta - \beta_\alpha$ site with the β L223. The general presence of multiple hydrogen-bonding partners makes the Sevoflurane very mobile in the site.

4.0.5 Different binding sites are hydrated differently.

Figure 5 conveys that the different inter-subunit binding sites differ in the number of water/lipid molecules occupying the site in Apo receptor and the number of molecules displaced by binding of propofol to receptors. One of the key observations is that the $\alpha_\gamma - \gamma$ site has significantly more lipid occupancy as compared to the other sites. On close observation and as depicted in figure 5 (B) and (C) , presence of a smaller ILE residue at the $\alpha_\gamma - \gamma$ (') as compared to presence of a bulkier Methionine residue at other interfaces, leads to increased exposure of the site to lipids, thus making it an open cavity.

4.0.6 Binding affinity directly correlates with increased water displacement in site.

Figure 6 (A) A depicts the direct correlation between the number of water displaced and binding affinity for propofol. In the apo receptor, the sites containing α and β subunits have similar number of water molecules. $\gamma - \beta_\gamma$ site has slightly more number of water molecules compared to the other sites due to the additional polar residue in site as shown in our previous study (45). Simulations reveal some water molecules being replaced due lipid binding in site $\alpha_\gamma - \gamma$. In order for an Anesthetic to bind the intersubunit sites, it would have to replace the water/lipid residues in site. Therefore the affinity of particular site would depend on the number of water/lipid residues removed or existing in that site following the binding of propofol. Figure 6 (B), shows the correlation between the affinity and the number of water/lipid atoms existing in site. We see the affinity increases with ability of propofol being able replace all the water molecules in site.

5 Conclusion

Molecular dynamics simulations of *Propofol- $\alpha_1\beta_3\gamma_2$* identified stable Propofol binding conformations in GABA_AR.Flooding simulations revealed intrasubunit sites and multiple occupancy in intersubunit site for Sevoflurane. Based on the EC₅₀ and calculated affinity of propofol and sevoflurane,3 intersubunit sites, $\alpha_\beta - \beta_\alpha$, $\gamma - \beta_\gamma$ and $\beta_\gamma - \alpha_\beta$, would be occupied by propofol at clinical concentration; 3-5 intersubunit sites being occupied by Sevoflurane at clinical concentration. From calculating the correlation between the binding affinity was water/lipid displacement, binding is most favorable when water is displaced and least favorable when lipid is displaced by the anesthetic Contrary to expectations, persistent hydrogen bonding between propofol and receptor is not necessary or sufficient for a higher affinity site, although we do observe a weak trend.

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