

A . Project Objectives:

Ion channels are a class of proteins that form pores in the cell membrane for movement of ions down through their concentration gradient. The openness or closure of an ion channel depends on the potential difference across the cell membrane, binding of a ligand to the channel, pH, pressure/stretch, etc. A significant class of channels activated by a ligand are called pentameric ligand gated ion channels(pLGIC). Depending on the charge of ions conducted, these receptors/channels contribute to inhibitory or excitatory processes in the central nervous system(CNS). Malfunction of the ion channels due to mutations or agonists/antagonists lead to many defects like epilepsy with seizures or sedation and coma, depending on the activation or inhibition of these receptors. Many naturally occurring compounds like neurosteroids and man-made drugs like anesthetics are known to modulate these receptors by unexplained mechanisms. Thus the knowledge of the gating mechanism is extremely important to consider ion channels as drug targets and to control various critical processes in our body. My objectives towards understanding the mechanism of modulation and gating in these channels are:

- 1. To identify and explain the effect of a disease associated mutation in GABA_A receptor.**

The GABA_A receptor, an anionic eukaryotic pLGIC, is known to cause febrile seizures in the presence of a mutation K289M in the $\gamma 2$ subunit. The exact mechanism by which the mutation leads to the closure of the channel is presently unknown. Also, the knowledge of this significant change brought by the mutation is thought to provide insights into the gating mechanism or conduction of the channel. These questions will be answered by com-

paring, the dynamics of the wild-type and mutant receptor, using Molecular dynamic(MD) simulations and free energy changes for translocation of an ion thorough the channel,using Adaptive biasing force methods(ABF).

2. To recognize the role of aromatic residues in the gating of the channels, GLIC and ELIC.

ELIC and GLIC are prokaryotic, ligand gated cation channels. It is not known how the differences in the presence and absence of aromatic residues in the M4 helix of the transmembrane domain(TMD) , cause an effect in the gating of the channel. This will be investigated by performing MD simulations of GLIC and ELIC with deletions and incorporations of certain aromatic residues, respectively.

3. To analyze isolated binding sites of certain anesthetics, neurosteroids and thyroid hormone in GABA_A receptor and nicotinic acetylcholine receptor(nAChR).

Many binding sites for anesthetics, neurosteroids and thyroid hormone have been reported through experiments, in the intersubunit, intra-subunit and pore regions of these channels. The specificity and the binding affinity of the modulators to these sites are to be determined. The changes in the mode of action of these anesthetics depending on the sites of action , also remains unanswered. We will use docking techniques to identify initial coordinates of the ligand in the site. Following this the drug-receptor complex will be

simulated using MD. The binding affinities of the modulators will be calculated using Alchemical free energy perturbation(AFEP).

B. Background and Significance:

Pentameric ligand gated ion channels (pLGICs) pLGICs, also known as Cys-loop receptors, are transmembrane proteins that allow ions to pass in and out of a cell, in response to binding of a chemical messenger (ligand), including the neurotransmitters. These receptors carry a characteristic disulphide bond between the two cysteine residues in the N-terminal domain and are further classified based on the ions they conduct. Cationic channels, that include nicotinic Acetylcholine receptors (nAChRs), (5-HT₃) Serotonin receptors, and prokaryotic channels, ELIC and GLIC, conduct cations like N⁺, K⁺ and Ca²⁺. Anionic channels that include gamma Amino-butyric acid receptors(GABAAR), Glycine receptors and Glutamate-gated chloride channel(GluCl) , conduct Cl⁻ ions. High resolution structures are available for the eukaryotic homolog GluCl, medium resolution structures from cryo Electron microscopic images for nAChRs[N Unwin 2005] as well as several crystal structures for the prokaryotic homologs, GLIC [N Bocquet et al. 2009] [R Hilf 2009] and ELIC[Ricarda J. et al 2008]. These structures showed that the pLGICs, consist of five subunits differentiated as two main domains, the extracellular domain(ECD) and the transmembrane domain (TMD). Some eukaryotic receptors (Figure 1) are also known to have an intracellular domain, but the structure is yet to be determined. Each of the five subunits comprises of four helices in the TMD named as M1-M4, with M2 as the pore lining helix (Figure 1).

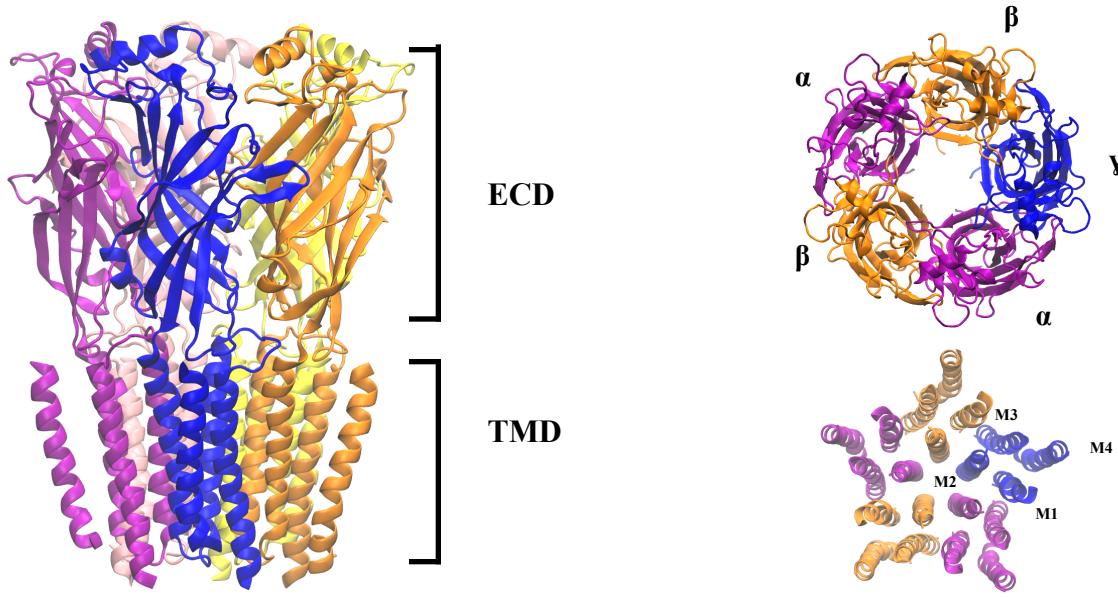


Figure 1: (Left) Depicts the side view of the two domains of the receptor, Extracellular domain and Transmembrane domain.(Right) Depicts the top view of the arrangement of different subunits around the pore the four helices of each subunit(M1-M4) in ($\alpha\beta\gamma$) GABA_A receptor.

GABA_A Receptor GABA is the primary inhibitory neurotransmitter in the central nervous system; inhibition is partially transduced by extracellular binding to the type A GABA receptor. Many molecules with sedative, anxiolytic, and anesthetic properties are positive modulators or agonists of the GABA(A) receptor, including neurosteroids[Lambert JJ et al. 1995], benzodiazepines[Costa E et al. 1991], and inhalational and intravenous general anesthetics [Pearce et al. 1998]. Negative modulators and antagonists, such as pregnanolone sulfate, bicuculline and picrotoxin can induce seizures, as can certain mutations. Being a heteropentamer, the receptor has its subunits drawn from different families (α , β , γ , δ , ϵ , Π , θ), with each family having its own subtypes. The most commonly found isoforms are $\alpha_1\beta_2\gamma_2$, $\alpha_1\beta_3\gamma_2$ and $\alpha\beta\delta$. The binding site of GABA is between the alpha and beta subunit (ECD), while the benzodiazepine binding site is between the alpha and gamma subunit (ECD). Neurosteroids and anesthetics are expected to bind to TMD.

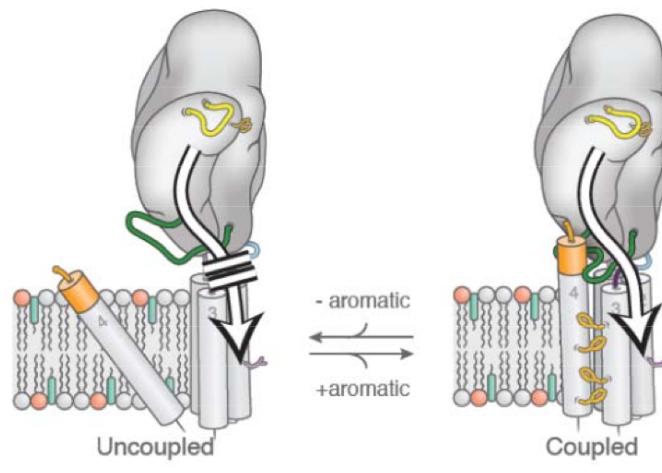
The M2-M3 loop , connecting helices M2 and M3 and interfacing with ECD, is considered as an important secondary structure that contributes in channel gating [Bera, AK et al 2002]. Specifically, a lysine residue in this region of the α 1-subunit is observed to form a salt bridge with the amino acid Aspartic acid, during the process of gating, in GABA_A receptors.[Thomas L. Kash et al 2003].Thorough inspection of this region led to the discovery of a molecular pathway connecting the binding site to the top of the M2-domain. In nAChRs, the binding of the agonist and the consequent events are transmitted to the entire channel through the electrostatic interactions between two residues forming a salt bridge.[S M.Sine et al. 2006].The importance of such salt bridges has been inferred from the fact that charge reversal of either of the residues forming the salt bridge impairs the channel gating. Mutations of these conserved residues are therefore likely to alter the channel's gating mechanism.

K289M Mutation K289M is one such mutation, which has been reported in families with generalized epilepsy and febrile seizures(fever caused seizures) [Baulac et al. 2001]. K289 is located in the M2-M3 loop of the γ 2 subunit.Several potential effects of this mutation have been identified.The consequences of the occurrence of this mutation have been analyzed in the past studies and have still been controversial.In α 1 β 2 γ 2(K289M) receptors, GABA-evoked current amplitude was dramatically reduced relative to the wild type while in α 1 β 3 γ 2(K289M) receptors the mutation did not affect current amplitudes but did increase the deactivation rate [Bianchi and song 2002] [Hales et al 2006].In the latter receptors, currents had reduced mean open times, in part due to flickering [Bianchi 2002].

Lipid Interactions In nAChRs the AChR-lipid interactions are considered as an important aspect of study because the receptors are continuously exposed to the lipids starting from its syn-

thesis to its final destination [F J Barrantes 2004]. Subsequent studies on individual lipid species have shown that cholesterol and phosphatidic acid stabilize the proportion of agonist-activatable form in nAChR [Baenziger J E et al. 2000] [Hamouda A K et al 2006]. Of the four TM helices, M4 is the most exposed to the lipid membrane, and is thought to be a site for lipid sensing [Antollini et al. 2005] [Williamson et al. 2005] [Xu et al. 2005]. Furthermore, a key structure connecting the C-terminus of M4 helices to the cys-loop has been identified , thus demonstrating a possible link between agonist site and channel gating [Jha et al. 2007][Lee et al. 2009]. A probable hypothesis obtained from these studies has been that activated lipids could enhance M4 binding to M1/M3 helices which in turn could stabilize the post-M4/Cys-loop interactions [daCosta and Baenziger, 2009][daCosta et al. 2013]. Consequently it has also been shown that any mutation in the M4 helices, that affects M4/M1/M3 interactions , seems to have an effect on the allosteric path leading from the agonist site to the channel gating [Tamamizu et al. 2000] [Shen et al. 2006].

Figure 2: A schematic of a single subunit showing the proposed role of M4 as an allosteric regulator of gating. The arrow denotes the allosteric pathway leading from the agonist site to the transmembrane gate. “Ineffective” interactions between M4 and M1/M3 are represented by a tilting of M4 away from M1/M3. Aromatic additions and deletions are used here to modulate the binding of M4 to M1/M3. [J E Baenziger et al]



Since it has already been proven that aromatic residues play a crucial role in engaging the binding of M4 to M1/M3 during folding in Glycine receptors [Haeger et al. 2010], the hypothesis (formed by collaborators at the university of Ottawa) is that the aromatic residues could influence the same even in the folded state. On comparing the sequence and structure of GLIC and ELIC, it has been observed that the four aromatic residues in the post-M4 cluster and two of them near the bilayer center, present in GLIC, are absent in ELIC. This absence of aromatic residues is proposed as contributing to insensitivity of ELIC towards agonists. Insertion and deletions of the specific aromatic residues in the ELIC and GLIC has shown gain and loss of function, respectively; however, the likelihood that this reflects proposed structural changes is unknown.

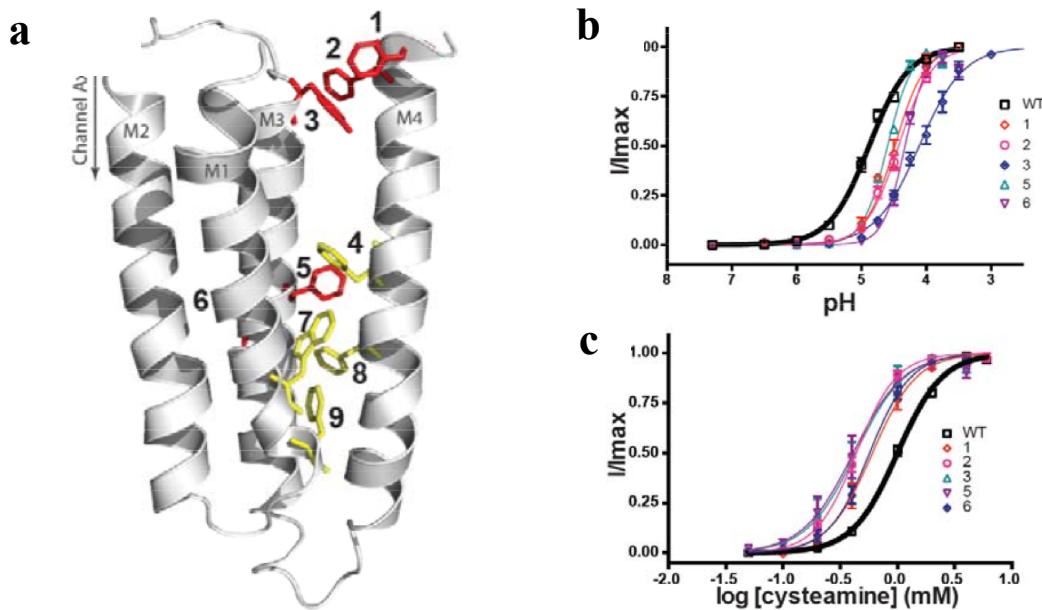


Figure 3: (a) Depicts the aromatic residues that are absent in ELIC, while present in GLIC(red). (b) Effect of the individual mutation on the current for GLIC. There is a right ward shift in the curve, showing the decrease in activity. (c) Effect of the individual mutation on the current for ELIC. There is a left ward shift in the curve, showing the increase in activity.[J E Baenziger et al.]

Thus, reproducing a similar setup computationally and analyzing their modulation might provide insights into the mechanism of gating of these channels.

General Anesthetics General Anesthetics are small molecules that induce immobilization, unconsciousness and amnesia by depressing neuronal signaling [Collins et al 1995][Fujiwana et al 1988]. Anesthetics, initially thought to bind only to the lipid membrane [Meyer 1899; Meyer 1901; Overton 1901], later, based on x-ray and neutron diffraction studies, were also found to bind to proteins[Franks and Lieb 1994]. Studies have also shown that anesthetics have multiple sites of action in the ion channel and its mechanism depends on the cell type of the target and the concentration applied to the target[Belelli et al. 1999]. Defining the binding sites for volatile anesthetic agents has been challenging due to its low affinity, volatility and rapid binding kinetics. In addition to the NMR and electrophysiological studies, a novel technique, photoaffinity labeling is being used for understanding the binding sites.[Echkenhoff RG , Shuman H 1993].

Classification Anesthetics can be broadly classified into two categories as local anesthetics that causes a reversible loss of sensation for a limited region of the body while maintaining consciousness and general anesthetics that causes reversible loss of consciousness. Depending the route of administration, the general anesthetics are further classified as intravenous and inhalational anesthetics. As the name suggests, inhalational anesthetics are volatile in nature and isoflurane, desflurane, sevoflurane and nitrous oxide are the volatile anesthetics in current use. Propofol is the primary intravenous general anesthetic used clinically.

Identified Binding sites on the GABAA receptor Most general anesthetics cause gain of function in GABAA receptors at clinical concentrations. Initially, with the lack of a crystal structure, mutagenesis studies have been crucial for detecting binding sites in the GABAA receptors and nACh receptors. Several amino acids in the TMD, at the intra-subunit regions of $\alpha(\alpha 2$ S270, $\alpha 2$ A291) and $\beta(\beta 1$ S265, $\beta 1$ M286) have been shown to be involved in the positive modulation of GABAA receptor by general anesthetics by mutagenesis [M Krasowski et al. 1998][K Nishikawa et al. 2002] [G S Findlay et al. 2000] . This intra-subunit site at the $\alpha 2$ and $\beta 1$ subunit, upon mutation has been shown to cause increased sensitivity to agonist and insensitivity to isoflurane and other intravenous anesthetics [M Krasowski et al. 1998] and positive modulation of the receptor [G S Findlay et al. 2000]. Further studies showed that the mutation of the residues involved in the $\alpha 2$ subunit affected the dose-response curve of GABA in the presence of anesthetics, halothane, isoflurane[K Nishikawa et al. 2002] and completely abolished potentiation of receptor in presence of sevoflurane and desflurane[K Nishikawa et al. 2003]. Homologous to $\alpha(\alpha 2$ S270, $\alpha 2$ A291), sites were identified in $\alpha 3$ subunit(S294 and A315), which respectively, decreased or inhibited modulation by isoflurane upon mutation.[C Schofield et al. 2005]. Photolabelling studies have identified a novel inter-subunit binding site ($\alpha 1$ M236 - $\beta 3$ S286) for etomidate in the GABAA receptor, with an etomidate analog [G Dong Li et al. 2006]. This site , further analyzed with for propofol and isoflurane, showed that, while the etomidate photolabelling was partially inhibited by propofol, it was completely abolished in the presence of isoflurane. This study concluded saying that isoflurane bound to the same site as etomidate analog and propofol allosterically modulated the binding of etomidate analog.[G Dong Li et al. 2010]. Photolabelling studies with an improved etomidate analog captured the involvement of

β 3(V290) in addition to the already known α 1 M236, in the formation of the intersubunit binding site[D Chiara et al. 2012]. Two other novel at intersubunit interfaces of α - β and β - γ subunits have been identified using an etomidate analog. Structurally heterogenous anesthetics, propofol, etomidate and barbituric acid was shown potentiate the receptor when bound to this site , but with different selectivity [D Chiara et al. 2013].

Identified Binding sites on nAChR Most general anesthetics inhibit nAChRs at clinical concentrations. Photo affinity and NMR studies have contributed to identifying the binding sites in nAChr. nAChr is one of the most analyzed ion channels using photo labeling techniques. Halothane and etomidate analogs have been largely used to identify binding sites. Initially photo labeling experiments were useful in demonstrating that anesthetic binding sites are not identical but similar in character.[R G Eckenhoff et al. 1996]. Etomidate analogs have been used to identify binding sites at two different locations, with different mechanism of action. The etomidate analog functions as a positive modulator at the α - γ interface in an intersubunit site and as an inhibitor when bound to the lumen of the channel[S Nirthan et al. 2008]. In contrast, a similar experiment with a different etomidate analog showed that the analog, when bound to the α - γ interface, acted as a negative modulator and did not inhibit channel blockers in the pore region [A K Hamouda et al. 2011]. Similarly etomidate analogs have been used to identify amino acids involved in an intra-subunit(δ) binding site, extracellular end of the ion channel and a site in the TMD[David C Chiara et al 2009]. Recently, an analog of propofol was used and it was shown to bind in the δ subunit, in the ion channel and at the γ - α interface. It has also been observed that propofol inhibits photo labeling at the site inside the ion channel and potentiates the analog at γ - α interface [Selwyn S Jayakar et al. 2013]. NMR studies have also payed a critical role in identi-

fying binding sites in nAChR. NMR studies have shown that in α 4 β 2 receptors, the anesthetic isoflurane induces lipid heterogeneity , thereby causing rotational and tilting motion in the helices, which in turn modulates the receptor [T Cui et al. 2010]. Similarly, the α 7 β 4 receptor has been shown to have an intersubunit site for isoflurane, that brings about a change in the dynamics of the gating residue α (L249) , thus causing inhibition of the receptor [D Mowrey et al. 2013]. A recent NMR study with the α 4 β 2 receptor was performed in the presence and absence of anesthetics ketamine and halothane. Both the anesthetics were found to bind an intra-subunit cavity of the β 2 helix and near the selectivity filter at the intracellular end of the TMD, in addition to Halothane being found to bind to an intersubunit interface of the α 4 β 2 receptor[V Bon-darenko et al. 2013].

Challenges Identifying the location of binding sites for anesthetics is of prime importance for understanding the mechanism of their action and for designing better drugs. Techniques like NMR, photoaffinity labeling and electrophysiology have been crucial in identifying general anesthetic binding sites. While photoaffinity labeling can report the amino acids involved in the binding sites, mutagenesis studies combined with electrophysiology, can also explain the effects of the presence and absence of these amino acids in the binding pocket. Though such studies have revealed considerable information, they fail to provide a complete picture of anesthetic binding sites, because the choice of mutations or selective reactivity of the photolabel prevent the whole receptor from being explored. In addition, such techniques are only able to indirectly infer the spatial location of the binding sites from the identified region of amino acids. Exploring these experimental results with the developing computational techniques can serve to directly provide insights into the microscopic features of anesthetic-ion channel interactions.

Computational techniques Advances in computational techniques have offered great opportunities in exploring protein structure and dynamics, as well as ligand binding. One such key technique is docking, in which a macromolecule is searched for pockets or cavities that could accommodate a given ligand. Several scoring functions are available for predicting the strength of non-covalent interactions thus providing an estimate for the relative binding affinities of different poses. Auto-dock is an automated tool for docking ligands to proteins of known three dimensional structure [Huey, R., Morris et al. 2007]. Docking results combined with Molecular dynamic(MD) simulation studies can illuminate the influence of drug/ligand on the protein. An alternative to the docking technique is “flooding”, in which the drug/anesthetic is placed in the surrounding water around the ion channel and allowed to partition into lipid and protein binding sites over the course of a molecular dynamics simulation. Obtaining rigorous estimates for binding affinities from such MD simulation studies is done by a technique called alchemical free energy perturbation(FEP). Such techniques have helped in overcoming many shortcomings faced by studies using the previously mentioned experimental techniques.

Computational studies Combined with experimental results, information from computational work helps us identify and validate critical binding sites. Docking followed by MD simulations have been used to analyze the experimentally found binding sites for isoflurane in GLIC [D Wilenbring et al. 2011] and halothane in nAChR [Liu LT et al. 2009]. MD simulations have also been used to identify altered motions and significant changes in critical regions of the receptor GLIC in the presence of the halothane [Chen Qiang et al. 2010]. Comparing the differences in modulation of wild-type and mutant GLIC has led to observing changes in tilting of TM helices leading to insensitivity towards the anesthetics propofol and etomidate [T Tillman et al. 2013].

Flooding simulations have been useful in unbiased search of binding sites for isoflurane in GLIC [G Brannigan et al. 2010], for halothane in closed nAChR [Liu LT et al. 2009]. FEP calculations can provide the absolute binding affinities for isolated sites, instead of averages over the entire receptor. FEP has been used for comparing the differences in the binding affinities of similar sites in open and closed nAChR [Liu LT et al. 2009], to analyze the pore block mechanism of isoflurane in GLIC [David N Lebard et al. 2010]. Recently, binding affinities of propofol docked to a homology model of GABA_A receptor has been shown to have linear correlation with GABA_A receptor potentiation EC50. Various such studies which has been experimentally improbable or expensive have been made possible by the recent advances in computational techniques.

Neurosteroids Neurosteroids synthesized in the central and peripheral nervous system from cholesterol precursors have an effect on neuronal excitability through their interactions with ion channels, especially GABA_A receptors. Naturally occurring steroids such as allopregnanolone and tetrahydrodeoxycorticosterone have been surmised to enhance GABA-mediated chloride currents, whereas pregnenolone sulfate and dehydroepiandrosterone (DHEA) sulfate show antagonistic properties at GABA_A receptors. Neurosteroids and Thyroid hormones(T3) have been proposed to modulate GABA_A receptor by a similar mechanism [Martin J V et al. 2004]. Recently , voltage-clamp studies revealed that T3 shows competitive binding effects with both allo-pregnanolone and ivermectin bound to GABA_A receptors, which is interpreted to indicate overlapping binding modes (presumably at the subunit interface) among these three molecules. [T Westergard et al. 2014]. However, affinities of the molecules for the intersubunit sites have not yet been calculated.

C. Preliminary Studies

GABA_AR Model There are no high resolution structure available for GABA_A receptor but multiple structures for members of the same Cys-loop family have been published; for simulations of the GABA_AR, we must rely on homology models built on these templates. We used GluCl as the template for which the alignment with GABA_AR is essentially unambiguous and has no gaps. GluCl and GABA_AR (both anionic, eukaryotic, ligand-gated) contain 32% sequence identity and 46% identity in the transmembrane domain. At a temperature above 300K, most residues with conformations that vary across generated models will also visit multiple conformations over the course of the M simulation time, removing a significant amount of the initial bias and increasing uncertainty in measured quantities regardless of the initial model.

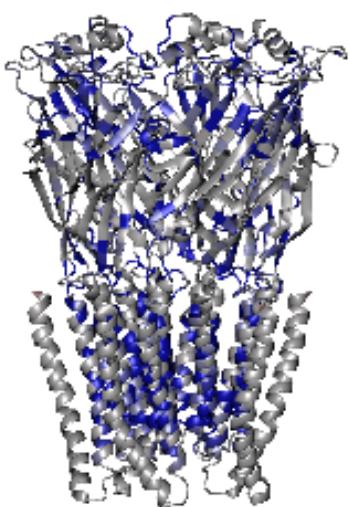


Figure 1: GABA_A receptor. The blue segments indicate the identical residues between the GABA_A receptor and GluCl receptor.

The models used in this paper correspond to Model 1 - CHOL from Reference [Henin 2014]. Details about the generations of these homology models built using GluCl (PDB code : 3RHW)[Hibbs R E et al. 2011] as a template , can be found in the **Section D: Computational Approaches**.

System Setup The systems were prepared as in Ref. [Henin 2014], by embedding the protein in a lipid bilayer composed of 4:1 phosphatidylcholine (POPC) : cholesterol mixture built using CHARMM Membrane builder [Vanommeslaeghe K et al. 2010], with the final system containing 268 POPC and 71 membrane CHOL molecules. In addition to membrane cholesterol, this model includes chole-

terol docked to five pseudo-symmetric intersubunit sites, with implications and justification for this decision reported in [Henin 2014] The systems were solvated (Figure 2a) using the SOLVATE plugin in VMD and neutralizing ions were added to bring the system to a 0.15M salt concentration using the AUTOIONIZE plugin (Figure 2b). The final system contained about 160,000 atoms (Figure 2c). All four systems were run at 300 and 315 K to understand the effect of temperature on the conformations of the receptor.

All simulations used the CHARMM22-CMAP force field [Humphrey W et al. 1996] with torsional corrections for proteins. The CHARMM36 model [Klauda, J. B. et al. 2010] was used for phospholipids , ions, and water. Energy minimization and MD simulations were conducted using the NAMD2.9 package [Phillips J. C. et al. 2005] (details can be found in the **Section D. Computational Approaches**). 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.

GABA_AR system All simulations were run in the NPT ensemble with weak coupling to Langevin thermostat and a barostat at a respective 300 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 the systems were energy minimized for 10000 steps, then simulated for 5 ns with restraints of 1 kcal/mol/Å applied to the C α atoms of the protein. Restraints were then removed and 195 ns of nearly unrestrained simulation was carried out in all four systems. During this period of the simulation, only harmonic restraints (force constant 0.4 kcal/mol/Å) between the intracellular ends of the M3 and M4 helices were used, to mimic the effects of the intracellular

domain and prevent separation of the M4 helix from the rest of the bundle.

This work consists of simulations from 2 replicas of wild-type and 2 replicas of Mutants. The mutation, from a charged(lysine) to uncharged residue (Methionine) (Figure 3), has been introduced in the M2-M3 loop of the γ 2 subunit using the VMD plug-in. The wild-type and the mutant structures were placed in a membrane surrounded by water and ions as depicted in the figure (2).

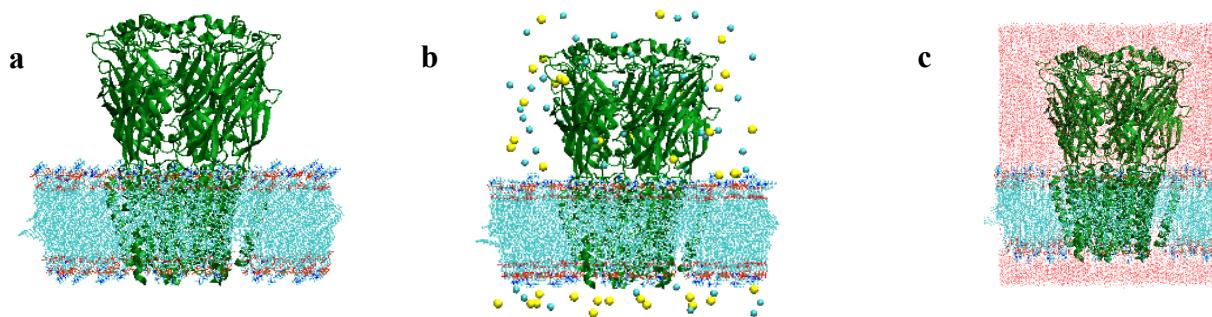


Figure 2: (a) Receptor with the lipid membrane. (b) Receptor in membrane with the Na⁺ and Cl⁻ ions. (c) Complete system with the water box.

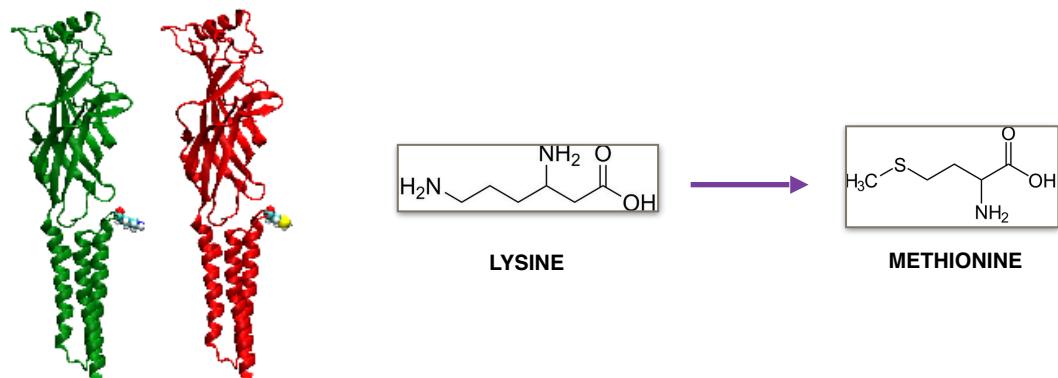


Figure 3: Gamma subunit (left) Wild-type with the lysine(K289) residue (right) Mutant with methionine (K289M)residue.

Pore radii analysis The transmembrane domain comprises of four helices(M1-M4) in each subunit and the M2 helices lines the pore of the channel. The M2 helices has an equal contribution of both polar(green) and non-polar (pink)residues as shown in the figure (4a). The charged residues (red and blue) are found only in the top and bottom of the helices. Whlie the residues facing the pore are dominated by the hydrophobic or non-polar residue as shown in the figure (4b).The minimum constriction in the pore is known to be formed by the Leusine residues as shown at the 9' region (Figure 4a).

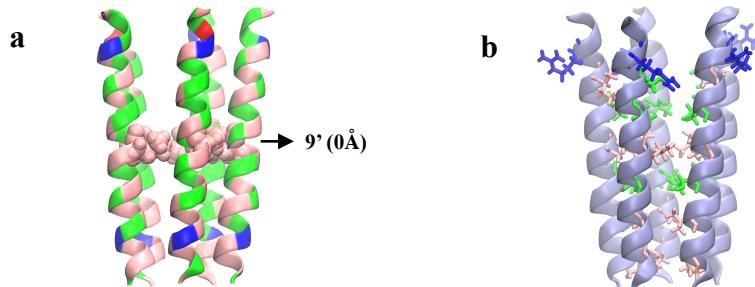


Figure 4: (a) M2 helices depicted with residues colored base on their type, polar(green), non-polar(pink), blue(basic) , red(acidic). The 9' position shows the ring of LEU residues depicting the minimum constriction of the pore. (b) Depicts the pore lining residues(colored by type of the residue).

The minimum pore radius at this region is $\sim 2\text{\AA}$ for an open channel. Thus, measure of the pore radii directly relates to the openness/closure of the channel.

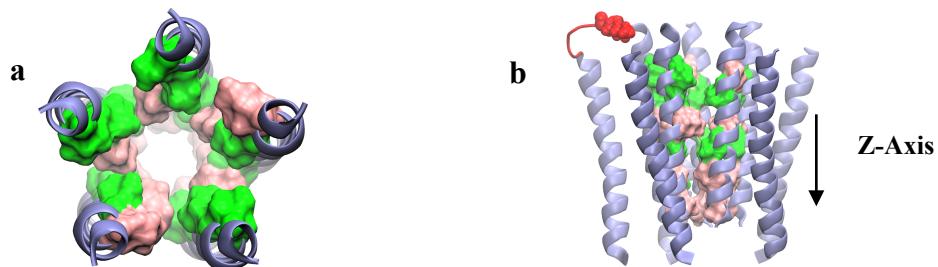


Figure 5: (a) Pore region , whose radii is measured using the pore radii script.(b) Depicts the proximity of the mutation to the pore region.

The effect of the mutation on the pore radii of the channel was measured using a TCL script in VMD [Humphrey W et al. 1996] and the HOLE software [Smart O. S et al. 1993] [Smart O. S et al. 1996]. The script measures the radii of the pore region traced by the pore facing residues as shown in the figure (5a).

The pore radii thus measured along the z axis as show in figure (5b) , shows that there is a significant reduction in pore radii in the mutants and this effect is more pronounced with increase in temperature ,consistent with the fact that the mutation K289M causes seizures with fever.

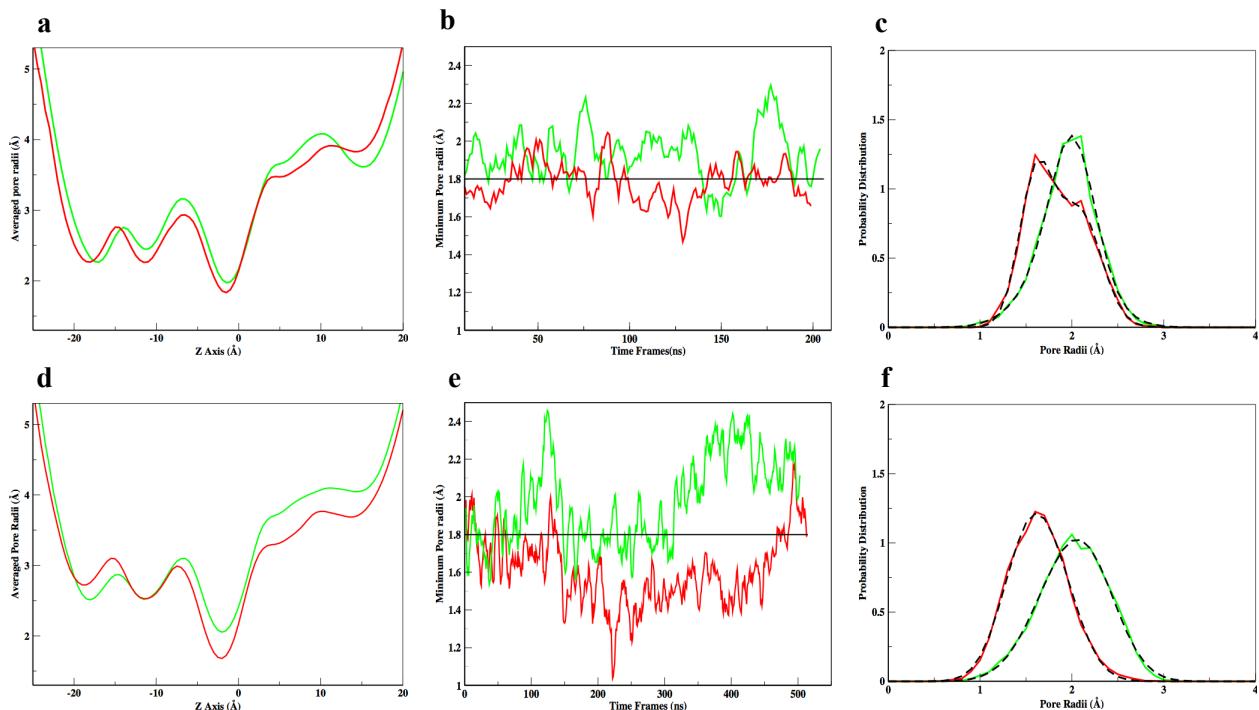


Figure 6: [Top 300K][Bottom 315K][Red-Mutant; Green-Wild-Type] (a),(d). Radii of the transmembrane domain along the Z-axis, averaged over all the frames. (b),(e). Smoothed time evolution of the pore minimum constriction, averaged over two replicas each. The line represents the radius of a Cl⁻ ion. (c),(f).Probability distribution of the minimum pore constriction.

It is also observed that the average radii of the minimum constriction falls below 2 Å, which is almost equal to the radii of Cl- ion. According to experimental studies the mutation is known to

cause a “flickering” effect that leads to fast conversions between conducting and non-conducting states [Bianchi 2002]. From figure (6a) ,(6b) it is evident that there is a significant reduction in the pore radii especially in the minimum constriction region (Figure 6(b), 6(d)) . Figure 6(f) and 6(c) ,further substantiates the effect of temperature, as the peak shifts to the left for mutant and remains the same for the wild-type with the increase in temperature. Furthermore, the peak has broadened for the wild-type (as expected) with increase in temperature while this is not evident in the mutants. The increase in distance between the peaks((Figure 6(b), 6(d))) of the mutant and wild-type and also the shift in the peaks could be consistent with the flickering effect.

Water in the pore Many theoretical studies on water have shown that the interfacial drying can be caused by hydrophobic enclosures in the protein [Fangqiang Zhu et al. 2012] . Furthermore, studies [Fangqiang Zhu et al. 2010] [H DOng et al. 2013] have also shown that drying of the pore region could lead to blocking of the channel, since water is assumed to facilitate conduction of ions.

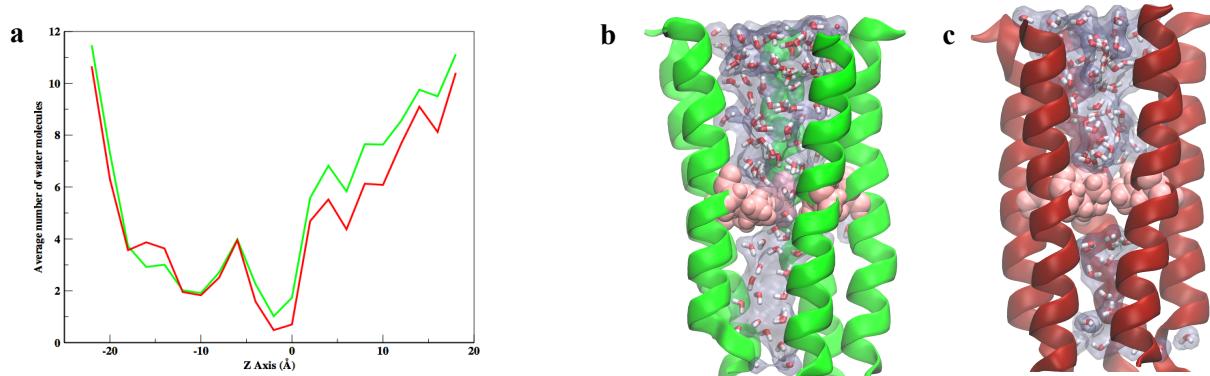


Figure 7: (a) Number of water molecules along the Z-axis averaged over the frames and replicas. (b) Presence of water in the constriction region of the wild type as compared to the temporary dryness due to reduction in pore radii in the Mutant(c).

As mentioned earlier, the pore facing residues in GABA_A receptors are dominated by non-polar residues and this causes intermittent drying of the channel when the minimum constriction region comes closer to form hydrophobic enclosures. The plot (figure 7a) shows the average number of water molecules throughout the simulation, along the Z-axis. The figure (7c) substantiates the plot by depicting the absence of water in the minimum constriction region of the pore, in the mutants . Thus , such dehydration of the channel could be a mechanism for inhibiting the conduction of the channel.

Electrostatic barriers in the channel Since the mutation involves a charged residue, it is reasonable to think that it might influence the electrostatic barriers of the channel. This led us to obtain the Poisson-Boltzmann(PB) electrostatic potential profile of the channel. This calculation, performed using APBS,explained in **Section D: Computational Approaches**, gives an idea about the electrostatic environment encountered by an anion as it travels through the channel.

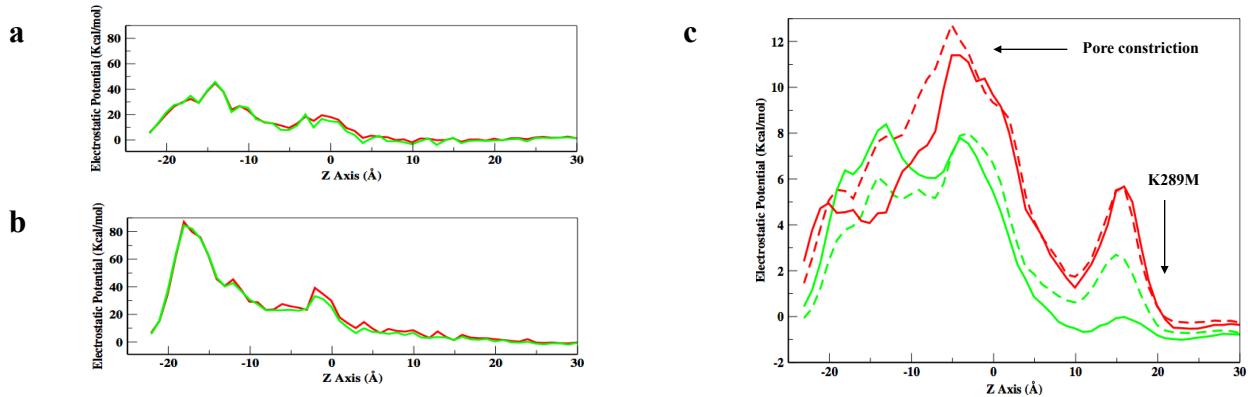


Figure 8: [Red -Mutant; Green - Wild-type] Electrostatic potential profile of the initial, non-equilibrated structure (a) Cl^- ion (b) Na^+ ion. (c) The Electrostatic potential profiles along the channel pore (for a chloride ion - averaged over 50ns for both the higher(--) and lower(—) temperature systems) The arrows indicate the region containing the mutation and the pore constriction in the channel.

The electrostatic interactions are long range interactions and thus are expected to have a significant effect throughout the channel. The plot on figure (8a,8b) represents the PB profile of the translocation of Cl⁻(figure (8a)) and Na⁺(figure (8b)) through the channel. These plots were for the initial non-equilibrated structure. The sodium ion faces much higher barrier as compared to the chloride ion, consistent with anionic nature of the GABA_A receptor. In spite of being non-equilibrated, the PB profile of the mutants show minute variations, compared to the wild-type profile, thus depicting the small effect of the mutation on the potential in the identical conformations. The figure(8c) shows the PB profile of the channel (TM domain) for Cl⁻ ion, at lower and higher temperature after equilibration of 200 ns and 400ns respectively, with the mutation. The mutant showed significantly higher barriers at these regions. The lysine(K289) residue in the wild-type, being a positively charged residue at the entrance of pore, could have created a favorable environment for the negatively charged ion to pass through and this in turn could have reduced the barriers; however this effect is only visible after the equilibration. This result can further be substantiated by calculating the free energy changes as the ion passes through the channel along the Z-axis.\, whig will include van der waals and entropic terms.

Free energy changes To calculate the free energy changes alone a reaction coordinate, the potential mean force (PMF) is to be calculated. We would undertake steered molecular dynamics(SMD) followed by Adaptive biasing force(ABF) methods to obtain PMF from the MD simulations as detailed in the **Section D: Computational Approaches**. Favorable positions of the ion along the channel were initially identified by performing SMD on the ion along the channel.

Plotting the average force experienced by the ion as a function of position in the channel , displays the barriers experienced by the ion along the Z-Axis. This result coincides with the previous results by conveying that the mutant experiences higher force at the minimum constriction region as compared to the wild-type.

The figure (9b) , depicting the movement of ion through the channel, also substantiates the hypothesis that partial drying of channel (figure (9b) - red) occurs at the minimum constriction region in the mutants. Thus the obtained positions of the ion along the z-axis in the pore can be

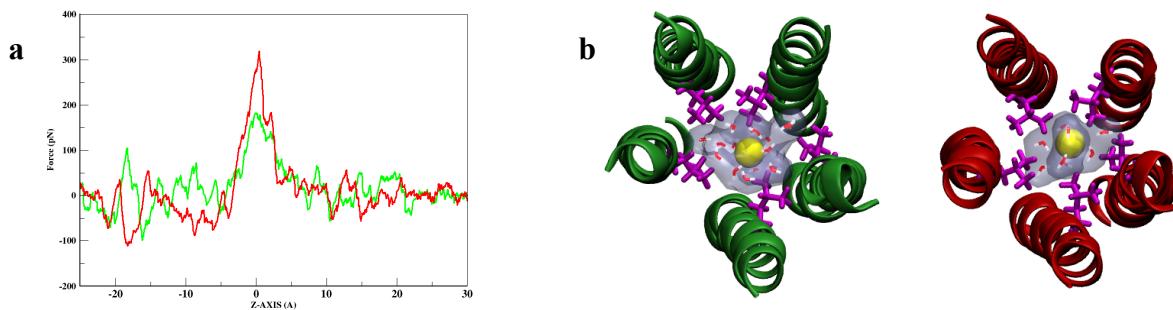


Figure 9: (a) The force as a function of position in the channel along the z axis(TM domain). (b) Snapshot from the SMD simulation depicting the Cl^- ion in the pore. (Green:wild type;Red:Mutant) Also shown is the reduction in water molecules around the ion in the mutant(red).

used as the initial structures for the proposed measurements of free energy changes in **Section E: Proposed Work**, using ABF .

Role of aromatic residues in the gating of ELIC and GLIC receptors Experience gained from working with GABA_A receptor has familiarized me with methods to visually and quantitatively analyze sometimes subtle , but important differences in the receptor and also the different techniques to interpret data from such modulations.

System setup The simulation system for the crystal structures of ELIC(3RQU) [Pan J et al.

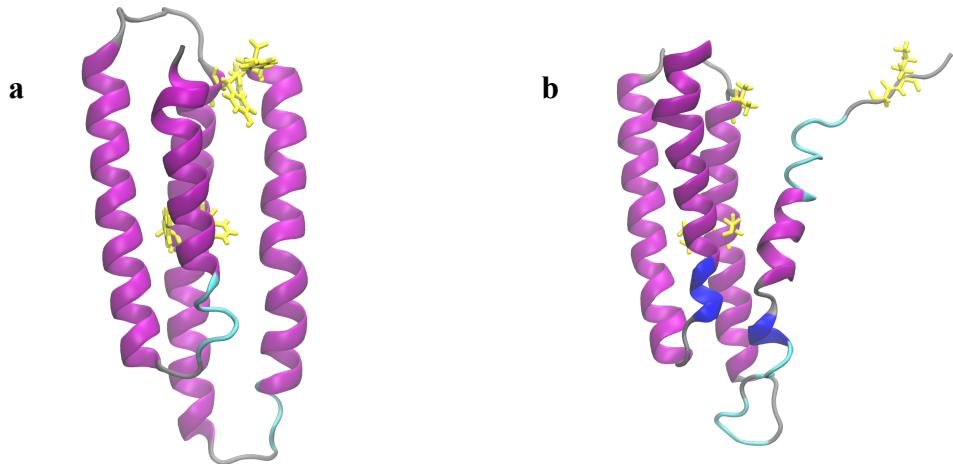


Figure 10: (a) GLIC (b) ELIC, colored based on their secondary structure, with residues of interest colored in yellow.

al. 2012] and GLIC(4HFI) [Sauguet L et al. 2013] with the resolution of 2.9Å and 2.4Å respectively respectively, was set up using the CHARMM-GUI [Sunhwan Jo et al. 2008]. The ELIC crystal structure lacked the structure or the C-terminal residues which were added using VMD. The ELIC system was then simulated with restraints (5kcal/mol/Å) on the entire protein except the newly added residues, for 5 ns, following which both the ELIC and GLIC systems are being equilibrated with restraints of 1kcal/mol/Å on selective residues to compensate for the absence of the neighboring subunits.

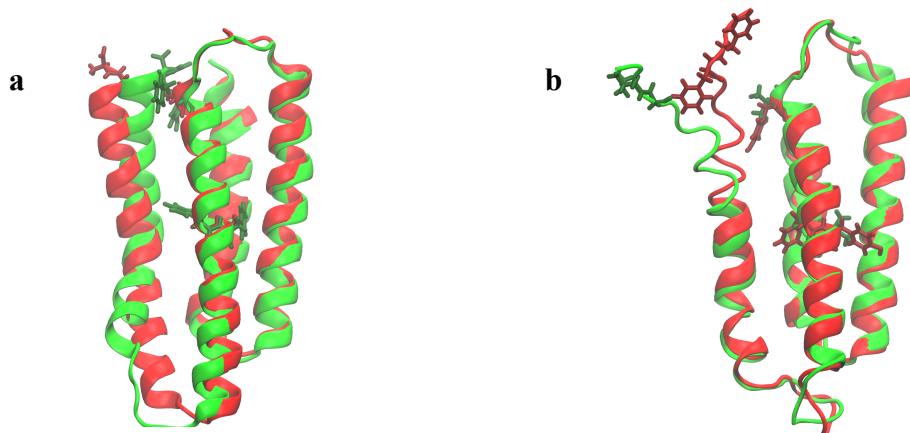


Figure 11: (a) GLIC (b) ELIC. Snapshots of the equilibrated structure of (green) Wild-type and (red)Mutant.

The mutation depicted in the figure(10a,10b) were introduction using the VMD plug-in MUTATE. The four systems (2 Wild-types and 2 mutants) were energy minimized and equilibrated employing the NAMD software, as explained in the **Section D: Computational Approaches**. The figure depicts the mutations incorporated in GLIC and ELIC receptors. The ELIC crystal structure has a kinked M4 helix as compared to GLIC. With the introduction of aromatic residues, the ELIC-M4 helix is hypothesized to straighten and bind to M1/M3 helices to facilitate gating, while losing the aromatic residues, GLIC-M4 helix is assumed to separate from M1/M3 helices and inhibit gating. This hypothesis is substantiated by the snapshot in figure 11, where the Mutant ELIC-M4 helix(Figure 11b) is more oriented towards M1/M3 helices while the Mutant GLIC-M4 helix shows inclination away from the M1/M3 helices (Figure 11a). With subsequent runs showing desired results, the simulations can be carried out with the whole receptor for the proposed work in **Section E: Proposed Work**

Effects of volatile anesthetics on pLGICs - GABA_A receptor and nicotinic Acetylcholine receptor

Experimental results have identified binding sites in the inter-, intra- subunits and the pore region of the pLGICs. The GABA_A receptor $\alpha 1\beta 3\gamma 2$ is being modeled and docked with MODELER and AUTODOCK(vina) as explained **Section D: Computational Approaches**. The figure (12) depicts the different sites for propofol in the GABA_A receptor identified by the docking software, which will be used as initial coordinates for the simulation work proposed in **Section E: Proposed Work**.

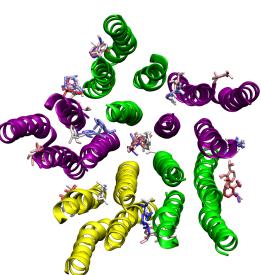


Figure 12: Propofol docked to the TM domain of GABA_A receptors.

D. Computational Approaches

Homology Modeling Homology Modeling is a technique , where an atomic-resolution model is created using the three-dimensional crystal structure of a homologous protein. Ten homology models of the human GABA_A receptor were built using the standard procedure for oligomer modeling in MODELLER [A. Sali & T.L. Blundell 1993] using the template and alignment, with disulfide bridges specified in the alpha and gamma transmembrane subunits. Stereo-chemistry was checked using Chirality plugin [Eduard Schreiner et al. 2011] in VMD and the model with the lowest score were chosen for further simulation.

Molecular Dynamics Molecular dynamics simulation is an approach for physics-based computer-generated simulation of atoms and molecules in order to calculate the time-dependent behavior of a molecular system. When applied to biophysical questions, simulation systems often contain proteins, nucleic acids, lipids, carbohydrates, solvent, ions, and/or small drug molecules. This tool helps in exploring the macroscopic properties of a system by altering the microscopic properties. This approach follows the Newton's second law of motion,

$$a_i = \frac{F_i}{m_i}$$

where 'F' is the force exerted on the particle 'i' and 'm' and 'a' are the mass and acceleration of the particle. In atomistic simulations, classical force-fields for covalent and non-covalent interactions are determined based on quantum calculations and experimental data; such force-fields include AMBER[Cornell WD et al. 1995] and CHARMM[Brooks BR et al. 1983]. After calculation of the forces exerted on the particles in the system, one can calculate the acceleration of the

particles in the system. Integrating these equations of motion allows one to obtain the positions and velocities of the particles. Several algorithms have been developed for integrating the equations of motion including Verlet [Verlet, Loup 1967], Leap-frog [C. K. Birdsall and A. B. Langdon 1985] and Beeman algorithms [Schofield, P. 1973]. Available softwares for generating trajectories include NAMD2[M. Nelson et al. 1996], which will be used in the proposed work. This is a parallel MD program developed by the Theoretical Biophysics Group at Illinois' Beckman Institute, available free of charge. In general, the systems are simulated in the isothermal-isobaric ensemble(NPT), where the system is maintained under constant pressure(P) and temperature(T) and the number of molecules(N) are also conserved, using techniques like Langevin dynamics [T Schlick (2002)].

Steered Molecular Dynamics SMD [S. Izrailev et al. 1998] is a method in which a constant force/velocity is applied to a specific part of the protein along its desired degrees of freedom. In our work, we had used a constant velocity of 10 Å/ns to pull the ion through the channel along the Z-axis, in a 15 ns simulation, for the purposes of generating initial coordinates for the later ABF calculations.

Adaptive Biasing force [E Darve et al. 2008] Comparison of the free energy profiles for an ion translocating an ion channel, between the wild-type and mutant form, can reveal the effects of the introduction of mutation on ion conduction. Calculating the free energy change along a chosen coordinate is called Potential of mean force (PMF). The Boltzmann distribution for free ion though the channel is,

$$P(z)\alpha \exp(-(A(z)/k_B T))$$

where $P(z)$ is the probability of finding an ion in the reaction coordinate z . ‘ z ’ represents the height of the ion channel along the z -axis. $A(z)$ is the Helmholtz free energy and k_B is the Boltzmann constant and T is the temperature.

From the above equation , free energy is given by the equation:

$$A(z) = -k_B T \ln P(z) \quad [1]$$

The change in energy with respect to the change in displacement along z -axis($Z_o - Z_i$) is given below.

$$A(z_o) - A(z_i)$$

But according to equation 1, the logarithmic relation between the free energy and $P(z)$ coordinate, means that even a small change in free energy may correspond to large changes in the value of $P(z)$ from its most likely value. But the normal MD simulation cannot explore the regions where the probability value($P(z)$) is highly unlikely. To overcome the errors due to inadequate sampling, we use Adaptive biasing force[M. Sprik et al. 1998][W. K. den Otter et al. 2000].

Differentiating the change in free energy with respect to change in Z -axis, we have,

$$\frac{dA(z)}{dz} = -\langle F(z) \rangle$$

The derivative of the free energy corresponds to the average force experienced by the ion. $F(z)$ is accumulated in small windows or bins of finite size, δz , thereby providing an estimate of the derivative defined in equation, which can then be used to reconstruct $A(z)$.

The force applied along the reaction coordinate ξ , to overcome free energy barriers is defined by:

$$F^{ABF} = -\langle F(z) \rangle$$

The biasing force introduced, thus ensures that the force experienced is nullified in each window. In the initial phase the force accumulated in each window takes very high and inaccurate values. Therefore the biasing force is generally applied after adequate number of force samples are collected in each window. After obtaining adequate samples, the free energy ($A(z)$) is reconstructed from overlapping windows. Implementation of ABF in our work is detailed in the **Section E: Proposed Work.**

Alchemical Free Energy Perturbations(AFEP) There have been multiple binding sites predicted for anesthetics and other modulators at the inter-, intra-subunit and pore regions of the ion channel. Measuring affinities for individual sites still remains unachievable by experiments. Obtaining the binding free energies of the anesthetics to the different sites is one way to differentiate between the binding sites. FEP [Pohorille A et al. 2010] [Woo H et al. 2005] [Gilson M K et al. 1997] is a theoretically exact computational technique that can be used to obtain absolute binding free energies from the MD simulations .

Considering a binding site with an anesthetic bound state(x) and unbound state (y), their free energy difference is given by,

$$\Delta A = A_y - A_x \quad [1]$$

But from statistical mechanics we know that,

$$A_x = -k_B T \ln Z_x; A_y = -k_B T \ln Z_y \quad [2]$$

K_B is the Boltzmann constant, T is the temperature and Z is the partition function, given by,

$$Z_x = \int d^n r \exp(-H_x(r)/k_B T) \quad ; \quad Z_y = \int d^n r \exp(-H_y(r)/k_B T) \quad [3]$$

This implies, from equation [2],

$$\Delta A = k_B T \ln(Z_y / Z_x) \quad [4]$$

From [3],

$$\frac{Z_y}{Z_x} = \frac{\int d^n r \exp(-H_y(r)/k_B T)}{\int d^n r \exp(-H_x(r)/k_B T)} \quad [5]$$

$$\frac{Z_y}{Z_x} = \frac{\int d^n r \exp((-H_y(r) - H_x(r))/k_B T) \exp(-H_x(r))}{\int d^n r \exp(-H_x(r)/k_B T)} \quad [6]$$

An average for a function $\langle f(r) \rangle$ is given by,

$$\langle f(r) \rangle = \frac{\int d^n r f(r) \exp(-H(r)/k_B T)}{\int d^n r \exp(-H(r)/k_B T)} \quad [7]$$

Therefore, we have,

$$\frac{Z_y}{Z_x} \langle \exp((-H_y(r) - H_x(r))/k_B T) \rangle_x \quad [8]$$

Substituting [8] in equation [4], we have,

$$\Delta A = k_B T \ln \langle \exp((-H_y(r) - H_x(r))/k_B T) \rangle_x \quad [9]$$

While Hamiltonian operators for both the bound and unbound state are to be calculated to obtain free energy, the trajectory is generated using the bound state. Convergence of the free energy is

still a problem due to inadequate sampling. Therefore the transformation from bound to unbound state is made gradual by introducing a coupling parameter λ ,

$$H_\lambda = (1 - \lambda)H_x - \lambda H_y$$

Using n series of windows with increasing λ_i , we have,

$$\Delta A_{\lambda_i} = k_B T \ln < \exp(-(H_{\lambda_{i+1}}(r) - H_{\lambda_i}(r)) / k_B T) >$$

By summing all the windows, we have,

$$\Delta A = \sum_i^n \Delta A_{\lambda_i}$$

Implementation of FEP in our work is detailed in the **Section E: Proposed Work**.

E. Proposed Work

Aim 1 : To identify and explain the effect of a disease associated mutation in GABA_A receptor. As explained in **Section B. Preliminary Work**, we are interested in seeing the effects of the mutation K289M on the dynamics of GABA_{AR}. Previous studies have shown that, there are several mutations occurring in GABA_{AR} that affects the expression levels, oligomerization of subunits , misfolding of the protein, etc. K289M is one of the few mutations that reduces open time of the channel and thus its effect is directly related to the mechanism of the channel. Unfortunately, it is experimentally challenging to analyze the dynamics of the protein at atomic resolution at physiologically relevant time resolution(nanosecond to microsecond time range). Further complicating this, is the absence of a crystal structure for GABA_A receptor.

Computational studies, on the other hand, allow for building a robust homology model of GABA_A receptor and performing in-silico mutation, using VMD plug-in (detailed in **Section B. Preliminary Work**). Further, with computational resources being available, one can perform MD simulation to compare and analyze the dynamics of the protein at higher timescales. Extremely long simulations would be able to capture the ions passing through the channel and the natural dynamics of the proteins can be visualized. But this would be computationally very expensive, and therefore there are techniques like SMD to apply forces on ions to pass through the channel at reasonable timescales. Furthermore there are also techniques like ABF to calculate the PMF experienced by an ion as it translocates between two coordinates in the ion channel. The initial phase of the work involves simulation of the GABA_A receptor with and without the mutation. 500ns of simulation data for each of the 2 replicas of Mutant and wild-type have been generated and the structural difference between the mutant and wild-type are investigated, namely by visualizing the changes in pore-radii and measuring the electrostatic barriers, as explained in Section C. Preliminary Work.

In addition, the effect of mutation on the ion diffusion through the channel is studied. Namely we employ SMD to create initial conformations for ABF and then construct the PMF for ion passage [techniques explained in **Section D. Computational Approaches**]. This will show the differences between the energy barriers for Cl in the two forms. With the initial and final coordinates as both ends of the channel along the z-axis, the coordinates would be split into 20 bins(5Å each). The ion's movement is constrained to the pore of the channel within the window and let to

explore the favorable minimum energy locations through extensive sampling. Following this, the biasing force would be applied to compensate for the energy barrier faced by the ion. 20-50ns of simulations would be run for each window before computing the PMF. Comparing the PMF plots of the Wild-type and the mutant would give insights into the contributions of the mutation on the dynamics of the proteins.

Aim 2 : To recognize the role of aromatic residues in the gating of the channels,

GLIC and ELIC. As explained in the **Section C. Background and Significance**, aromatic acids are observed to play a role in gating of the channel by improving the interaction between the M1/M3/M4 helices. Experimental studies have shown that the incorporation or deletion of the aromatic residues have improved and abolished gating in ELIC and GLIC respectively. But as explained earlier, analyzing the modulation differences caused by aromatic residues in the receptors ,through experiments would be challenging. We would employ MD simulations and VMD for analysis. ELIC and GLIC simulation systems containing only the TM domain of one of the subunits, after the incorporations of the mutations, are run using NAMD2.9 (details can be found in **Section D. Preliminary Work**). These results would be followed by running simulations with the whole receptor , with all the subunits incorporating the mutation. These results will help explaining the insensitivity shown by ELIC towards its modulators and also provide insights into the gating mechanism of these channels.

Aim 3 : To analyze isolated binding sites of certain volatile anesthetics, neurosteroids and Thyroid hormone(T3) in GABA_A receptor and nicotinic acetylcholine receptor(nAChR). As explained in **Section B. Preliminary Work**, various binding sites for the anesthetics , neurosteroids and thyroid hormone, have been found in GABA_{AR} and nAChRs. The sites are especially found in the TM domain, and are categorized as inter-, intra-subunit and pore regions. Determining the mechanism of modulation of the receptors by these modulators , would ultimately lead to providing insights into the gating mechanism of the receptors and would also lead to designing of better drugs targeting these receptors. These modulators are shown to bind to multiple binding sites and the only way to rank them is based on the binding affinity and specificity of the drug at the sites. Though experimental techniques have been able to predict the binding sites, acquiring the absolute binding affinities and visualizing of the modulation of a ligand at a specific site has been quite a formidable task. Comparatively, computational techniques have the advantage of being able to select specific binding sites for performing simulation with the modulators bound , using MD simulations. AFEP techniques, using all-atom explicit simulations, would calculate the absolute binding affinities of isolated sites, including all entropic and enthalpic contributions.

In Aim 2 we would implement AFEP as explained in the **Section D Computational Approaches**. Homology models of GABA_{AR} and nAChR will be created using MODELER. Two homology model would be chosen for each of the receptor. Selective volatile anesthetics, enflurane, isoflurane, desflurane, sevoflurane, intravenous anesthetic propofol , neurosteroids allopregnanolone and thyroid hormone T3, would be docked using AUTODOCK(vina) ,that uses multiple scoring functions. The search space would be restricted to the TM domain of the channels. The docking

tests would be rerun multiple times until all possible binding sites are explored. Of these specific binding sites would be chosen to carry out simulations using NAMD2.9. As described in **Section**

D Computational Approaches, in FEP simulations, the bound ligand is gradually decoupled from the channel over multiple windows (lambdas), by initially discharging the ligand and then turning off the van der waal's (VDW) interactions. The reverse FEP (re-coupling of the decoupled ligand) is also performed to evaluate convergence. Then the binding free energies of the ligand and for different sites are compared to rank their affinities. The quantitative results on the affinities of these different modulators will give insights on the following:

1. High affinity binding sites for each of the modulator.
2. Differences in the binding affinities of these modulators to the receptors.
3. Comparison of their mechanism of action on inhibitory(GABA_AR) and excitatory (nAChRs) receptors would help us compare the mechanism of gating in these receptors.

