The Dissertation Committee for Mandy Leigh McCracken Certifies that this is the approved version of the following dissertation:

EVIDENCE OF INTER- AND INTRA-SUBUNIT ALCOHOL AND ANESTHETIC BINDING CAVITIES IN THE GLYCINE RECEPTOR

Committee:

R. Adron Harris, Supervisor

Richard Aldrich

S. John Mihic

Richard Morrisett

James Trudell

EVIDENCE OF INTER- AND INTRA-SUBUNIT ALCOHOL AND ANESTHETIC BINDING CAVITIES IN THE GLYCINE RECEPTOR

by

Mandy Leigh McCracken, B.S.Psy.

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Dedication

To the millions of people personally afflicted by alcohol abuse and dependence and to the family and friends who have helplessly had to stand by and watch loved ones as they suffered the effects of this insidious disease, I dedicate this to you.

As scientists, we understand the reality of your challenges and we are committed to helping advance the development of improved treatment options through our pursuit of more clearly understanding the specific sites by which alcohol acts in the brain to produce intoxication.

Acknowledgements

As legendary coach Vince Lombardi said, “People who work together will win, whether it be against complex football defenses, or the problems of modern society.” Although this endeavor has not involved overcoming any complex football defenses, it has involved overcoming some unique “problems of modern society” while simultaneously negotiating the ordinary challenges associated with pursuing a doctoral degree in basic science. Fortunately though, I have been surrounded by a number of special people who have helped to make this possible by working with me so we could “win,” despite the unique “problems of modern society” that affected me personally at different points along the way.

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Although not formally a part of my dissertation committee, there are several additional people affiliated with The University of Texas at Austin (current or past) who I would like to acknowledge as well. Dr. Cecilia Borghese kindly provided me with much of the initial technical training, particularly in site-direct mutagenesis and two-electrode voltage clamp electrophysiology, I received when I began working in the Harris Lab as an undergraduate. I am infinitely grateful for the time she dedicated to helping me learn, and her continued interest in my progress to this day. Without the proteomic insight, technical expertise, and collaboration of Dr. Dayne Mayfield, Dr. Giorgio Gorini, and Dr. Yury Nuñez, the completion of the immunoblotting and mass spectrometry in this project would not have been possible. Their willingness to forge ahead and encouragement helped me to believe success was still possible, even in light of the many technical and experimental frustrations initially encountered. The enduring friendships of Drs. Gorini and Nuñez are also something I will take with me and value from this experience. Moreover, I am forever thankful for the guidance and urging of Dr. Deborah Stote, without whom I may not have ever found the Waggoner Center for Alcohol and Addiction Research or Dr. Harris, and consequently, had the opportunity to pursue to my true research interests and goals as both an undergraduate and graduate student. Furthermore, I am appreciative of the Harris Lab members, past and present, for creating a collaborative, collegial, and often social, work environment. I am the last of a "cohort" of graduate students in the Harris Lab who began at similar times, attended several courses together, and progressed through our respective program milestones one after another. We experienced most of this journey together, and I am thankful to them for

their wisdom as I finish this chapter of my education and move onto a postdoctoral position. To Dr. Chang Hoon Lee, thank you for your friendship and helping to keep me laughing and fed on a daily basis. To my twin sister Dr. Lindsay McCracken, thank you for keeping me continuously motivated and always challenging me not to be second best. I am also grateful to Debra James (past) and Jayna Dixon. Without their knowledge and skills, the Waggoner Center and Harris Lab would not run so efficiently, nor would it be as enjoyable a place to work.

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and accommodate my academic and career goals, especially the completion of this endeavor. It is due to the forging of our partnership that I have been physically able to continue my pursuit of a doctoral degree and a career in science. In addition, I have much gratitude for the contributions of Dr. Babak Larian in the Center for Advanced Head & Neck Surgery and Dr. Babak Azizzadeh in the Center for Advanced Facial Plastic & Reconstructive Surgery at Cedars Sinai Medical Center in Los Angeles. Their remarkable diagnostic and surgical skills as well as their first-rate bedside manner, especially over the last 9 months, have complimented my care at UCSF Medical Center and have given hope and provided improvement to what was an increasingly painful and frustrating situation. Lastly, the willingness of local physicians and surgeons in Austin to collaborate in my care has helped to reduce travel, and ultimately allowed me the time necessary on The University of Texas campus to complete this project. These individuals include: Dr. Vivek Mahendru and his associates at the Pain Specialists of Austin, especially Dr. Christopher Manees. Drs. Mahendru and Manees have worked tirelessly to help minimize my discomfort, but not with the total loss of my cognitive functioning. I am also grateful to plastic surgeon Dr. Jennifer Walden for her repeated willingness to collaborate with my other surgeons, especially those in California, to ensure that my surgical outcomes are both functional and as aesthetically pleasing as possible.

EVIDENCE OF INTER- AND INTRA-SUBUNIT ALCOHOL AND ANESTHETIC BINDING CAVITIES IN THE GLYCINE RECEPTOR

Mandy Leigh McCracken, Ph.D.

The University of Texas at Austin, 2014

Supervisor: R. Adron Harris

Alcohol is abundantly consumed by society and general anesthetics are used everyday in operating suites throughout the world, yet the sites and mechanisms of action for these drugs are not completely understood. Glycine receptors (GlyRs) are pentameric ion channels expressed throughout the brain and spinal cord and have become increasingly popular targets in the study of alcohol action. Each GlyR subunit is composed of four alpha helical transmembrane segments (TM1-4), and although amino acids involved with alcohol action have been previously identified in TM1-4, the orientation of each of these residues with respect to a putative alcohol/anesthetic binding cavity remains controversial. In order to better characterize this binding cavity within the GlyR, we conducted a series of experiments using cysteine mutagenesis and biochemical cross-linking. In Aim 1, the participation of TM1 with TM3 in a common alcohol/anesthetic binding cavity was further investigated. We used two-electrode voltage clamp electrophysiology in *Xenopus* oocytes to demonstrate the ability of A288 in TM3 to form cross-links with I229 in TM1, which reduced the ability of both alcohol and anesthetics to modulate GlyR function. Aim 2 investigated whether TM3 could also participate in a binding cavity with TM4. We have shown that residues in TM4 are able to form cross-links with A288 in TM3, and found that cross-linking between TM3 and

those residues in TM4 also reduced the ability of alcohol and anesthetics to enhance GlyR function. Aim 3 determined whether these cross-links are formed between residues within the same subunit (intra-subunit) or between subunits (inter-subunit), and ultimately whether these residues participate in a common alcohol/anesthetic binding cavity within or between GlyR subunits. GlyR protein, which measures about 50 kDa, was extracted from oocytes injected with the cysteine mutants, and immunoblotting was used with a GlyR-specific antibody to subsequently help quantify band ratios between cross-linked and uncross-linked conditions. We found an increase in the 100:50 kDa band ratio for the TM1-3 mutant only, but not TM3-4 mutant or the wild-type, which suggests TM1-3 may participate in an alcohol binding cavity between GlyR subunits while TM3-4 may contribute to a binding cavity within a subunit.

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CHAPTER I: Background and Significance

Despite the frequent consumption of alcohol in our society and the common use of general anesthetics in operating suites, scientists and healthcare providers are only beginning to understand how these drug molecules interact with molecular targets to produce physiological and behavioral effects. Alcohol, like anesthetics, is a CNS depressant capable of producing a range of dose-dependent physiological and behavioral effects beginning with low-dose disinhibition and anxiolytic properties followed by moderate-dose amnestic and other cognitive impairments. Finally, alcohol and anesthetics share similar high-dose end points including sedation, the induction of anesthesia, coma, and in severe circumstances, even death.

Originally, it was proposed that both alcohol and anesthetics exert their effects through action at lipid membranes (Janoff et al., 1981; Lyon et al., 1981), however a more current and widely accepted view is that these drug molecules act by binding directly to a number of proteins in the central nervous system (CNS), including glycine receptors (GlyRs) (Harris et al., 2008). GlyRs not only mediate the majority of inhibitory neurotransmission in the brain stem and spinal cord (Legendre, 2001), but they are also expressed in other brain regions including the olfactory bulb, hippocampus, nucleus accumbens (van den Pol and Gorces, 1988; Fatima-Shad and Barry, 1993; Molander and Soderpalm, 2005; Baer et al., 2009; Jonsson et al., 2012), and cerebellum (Takahashi et al., 1992).

THE GLYR STRUCTURE

GlyRs are members of the Cys-loop family of ligand-gated ion channels, which also includes the nicotinic acetylcholine receptor (nAChR), the serotonin 3 receptor (5-HT_3R), and γ-aminobutyric acid type A ($GABA_A$). Functional receptors are composed of five homologous subunits positioned around a central chloride channel, with the structure of each subunit consisting of an extracellular N-terminal domain, a transmembrane domain with four alpha helical segments (TM1, TM2, TM3, and TM4), an intracellular loop between TM3 and TM4, and an extracellular C-terminal domain (Figure 1.1). The glycine agonist binding site is located extracellularly at the interface between adjacent GlyR subunits, where pairs of oppositely charged residues on each side of the interface form optimal conditions for glycine binding (Grudzinska et al., 2005). The coupling of glycine agonist binding and channel gating has been proposed to involve an interaction of the TM2-TM3 linker region with the β_1-β_2 loop and the Cys loop (Kash et al., 2003). In addition, GlyRs can be gated by lower efficacy agonists such as taurine and β-alanine, however these agonists likely exert their effects through similar, but distinct, mechanisms from that of glycine (Pless et al., 2011).

Four GlyR α subunits (α1-4) and a single β subunit have been identified (Grenningloh et al., 1990; Harvey et al., 2000; Harvey et al., 2004). There is 80-90% sequence identity among the α subunits and approximately 50% identity among the β

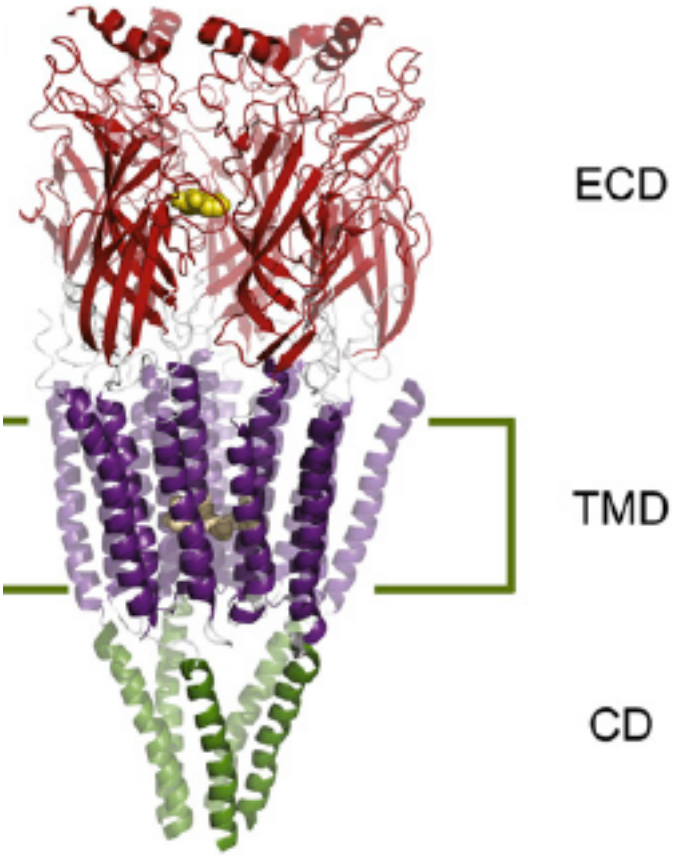

Figure 1.1. Representative 3D Structure of a ligand-gated ion channel. The extracellular domain (ECD) is depicted in red, transmembrane domain (TMD) in purple, and cytoplasmic domain (CD) or intracellular loop in green. In the absence of an x-ray crystal structure, GlyR homology models are built using related proteins with known sequences and structures as a template. The *Torpedo* nAChR was used as a template for the pentamer illustrated here (Adapted from Baenziger and Corringer, 2011).

subunit and the α subunits. GlyR subunits can assemble to form either homomeric α receptors or heteromeric αβ receptors (Lynch, 2004).

Although a complete high resolution GlyR crystal structure has not yet been solved, medium to high resolution structures exist for related ligand-gated ion channel motifs, including the acetylcholine binding protein (AChBP) (Brecj et al., 2001; Celie et al., 2004) and the *Torpedo* nAChR (Unwin, 2005). Additionally, the crystal structures for two prokaryotic homologues, Erwinia chrysanthemi (ELIC) and Gloebacter violaceus (GLIC) (Hilf and Dutzler, 2008; Hilf and Dutlzer, 2009; Bocquet et al., 2009), and the invertebrate Caenorhabditis elegans glutamate-gated chloride channel (GluCl) (Hibbs and Gouaux, 2011) have provided improved templates and better informed homology modeling for the study of GlyRs and related mammalian ion channels. Most recently, Mowrey et al. (2013) used full-length nuclear magnetic resonance (NMR) spectroscopy and electron micrographs to produce specific structural information from the expressed hGlyR-α1 TM domain. This lower resolution structural information, specifically from the GlyR, combined with the known homologue structures and experimental data from studies in heterologous expression systems should help to provide ongoing insight and more detailed, higher resolution information regarding GlyR structure.

THE GLYR, ALCOHOL, AND ANESTHETICS

Alcohols and volatile anesthetics were initially hypothesized to produce their effects through action at the lipid bilayer of cell membranes. This lipid hypothesis was

based on the early observation that volatile anesthetic potency strongly correlated with the ability of anesthetics to partition into olive oil (Meyer, 1899; Overton, 1901). More recently however, there has been accumulating evidence that these drug molecules instead bind to specific proteins (Franks and Lieb, 1984; Mihic et al., 1997), and currently, the protein sites of action theory is the predominant view (Harris et al., 2008). In addition, experimental evidence has shown that alcohols and volatile anesthetics likely share common sites of action in the GlyR (Mihic et al., 1997; Beckstead et al., 2001; Jenkins et al., 2001) and act as positive allosteric modulators (Harrison et al., 1993; Mascia et al., 1996).

Initial studies using heterologous expression systems, site-directed mutagenesis, and alkyl thiol reagents identified two critical amino acid residues for alcohol and anesthetic enhancement in GlyR TM2 and TM3. These include serine-267 (S267) and alanine-288 (A288) in TM2 and TM3, respectively (Mihic et al., 1997; Mascia et al. 2000). Additional experiments suggested that these residues along with isoleucine-229 (I229) from TM1 and others from TM4 likely participate in a common alcohol/anesthetic binding cavity (Jenkins et al., 2001; Lobo et al., 2004a; Lobo et al., 2006; McCracken et al., 2010).

More recently, Borghese et al. (2012) have also shown that mutation of residues neighboring the critical S267 site in TM2, glutamine-266 (Q266), and methionine-287 (M287) also eliminates alcohol enhancement of GlyR function. In addition to the aforementioned TM sites, other putative alcohol binding sites have also been proposed in the α1 GlyR, including alanine-52 (A52) in loop 2 of the N-terminal domain (Davies et

al., 2004; Crawford et al., 2008) and lysine-385 (K385) in the intracellular loop linking TM3 and TM4, which has been proposed to be involved in GlyR modulation by Gβγ (Yvenes et al., 2003; Yvenes et al., 2008).

CROSS-LINKING OF GLYR TRANSMEMBRANE SEGMENTS

Given that specific amino acid residues within the TM domain appear to be crucial for GlyR modulation by alcohols and anesthetics, investigating the proximity and orientation of these residues to each other as well as a putative alcohol/anesthetic-binding cavity is imperative in order to better understand alcohol and anesthetic action at GlyRs. The proximity and relative orientation of TM residues of interest can be investigated by substituting cysteine at each position, and subsequently testing whether or not they are able to form biochemical cross-links with one another. Residues of interest must be located on opposing helices in order to form cross-links, and typically their Cα carbons cannot be separated by greater than 15 A (Lee et al., 1995; Yang et al., 1996; Struthers et al., 2000; Soskine et al. 2002; Winston et al., 2005).

Due to the current absence of a GlyR crystal structure, there are multiple hypotheses regarding the site of alcohol- and anesthetic-binding within the GlyR TM domain. One hypothesis suggests that I229 in TM1, S267 in TM2, A288 in TM3 and others from TM4 may contribute a hydrophilic alcohol/anesthetic binding site within a GlyR subunit (intra-subunit). This intra-subunit hypothesis is supported by electrophysiology studies in heterologous expression systems using the substituted

cysteine accessibility method (SCAM) as well as initial cross-linking findings in GlyRs and homology modeling (Williams & Akabas, 1999; Yamakura et al., 2001; Lobo et al., 2004a; Lobo et al., 2004b; Lobo et al., 2006). In addition, x-ray structures of general anesthetics bound to the prokaryotic homologue GLIC support binding and modulation at an intra-subunit cavity (Nury et al., 2009). A second hypothesis has been proposed suggesting that the critical TM residues are positioned such that anesthetics instead bind at the interface between two adjacent subunits (inter-subunit). This inter-subunit hypothesis was first based on similar studies in the $GABA_A$ receptor to those described above in the GlyR (Bali et al., 2009) and has been recently supported by crystallography work paired with functional studies in GLIC which determined and compared the crystal structures of ethanol-treated and non-ethanol treated receptors (Sauguet et al., 2013). Finally, a third "multisite" hypothesis has emerged suggesting that perhaps alcohols and anesthetics may, in fact, bind and act at both intra-subunit and inter-subunit sites within these receptor proteins. This hypothesis has been generated largely from mutagenesis studies in the GLIC and GluCl homologues, molecular simulations, and newer homology modeling (Howard et al. 2011; Murail et al., 2012; Yoluk et al., 2013). In addition, recent structural information obtained from full-length nuclear magnetic resonance (NMR) spectroscopy and electron micrographs using expressed hGlyR-α1 TM domain demonstrated a considerable degree of flexibility at the extracellular end of the pore (Mowrey et al., 2013), allowing residue A288 in TM3 to possibly participate in both an intra-subunit and inter-subunit drug bind cavity in the GlyR. Recent cross-linking studies in the mammalian $GABA_A$ receptor also provide additional support for the "multisite"

hypothesis, but suggest that the effects may be influenced by receptor type and state as well as differentially affected by alcohols and anesthetics (Borghese et al., 2014).

GAPS IN OUR KNOWLEDGE

Despite accumulating evidence for the role of TM amino acid residues in alcohol and anesthetic enhancement of the α1 GlyR, several unknowns remain with regard to the contribution of these residues to a putative alcohol/anesthetic-binding pocket. First, in the absence of a GlyR crystal structure, the proximity and relative orientation of the critical TM amino acid residues involved with alcohol/anesthetic action has not been fully elucidated, and it is presently unknown whether the critical residues in TM1-3 contribute to an intra-subunit binding pocket within the TM domain of each GlyR subunit, form an inter-subunit pocket at the subunit interface as some have proposed in the $GABA_AR$ and shown in prokaryotic homologues, or alternatively, contribute to both an intra- and inter-subunit binding pocket (Figure 1.2). Moreover, because the involvement of TM4 in alcohol and anesthetic action has been much less studied than TM1-3, the significance of its contribution to a putative alcohol/anesthetic binding pocket remains less clear. This dissertation contains three aims that will address these areas and will contribute to a more thorough understanding of alcohol and anesthetic action at the GlyR.

SPECIFIC AIMS

1. To investigate whether TM1 participates in a common alcohol and anesthetic binding cavity with TM3.

2. To examine whether TM3 participates in a common alcohol and anesthetic binding cavity with TM4.

3. To determine whether the critical sites for alcohol and anesthetic action in TM1-4 are oriented to form a binding cavity within the same subunit (intra-subunit), between subunits (inter-subunit) or both.

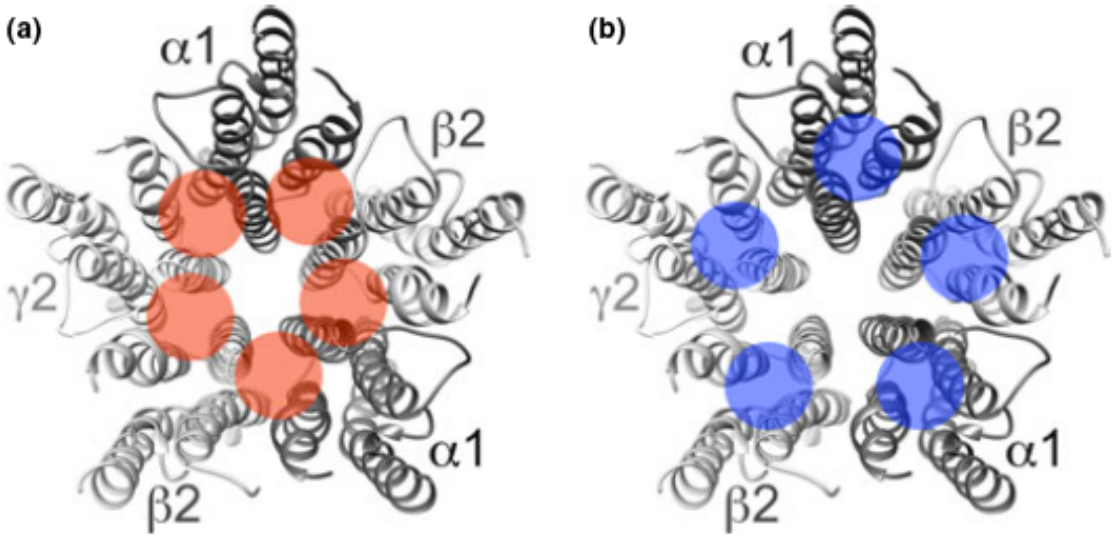

Figure 1.2. Putative alcohol and anesthetic binding sites. A) Inter-subunit cavities located at the interface between adjacent subunits are depicted in red on using a representative Cys-loop ion channel. B) Intra-subunit cavities are depicted in blue at the center of the helical bundle within each subunit (Adapted from Borghese et al., 2014).

CHAPTER II: Biochemical Cross-linking Provides Evidence that Glycine Receptor Transmembrane Segments 1 and 3 Participate in a Common Inter-subunit Alcohol and Volatile Anesthetic Binding Cavity

INTRODUCTION

Strychnine-sensitive glycine receptors (GlyRs), like other proteins in the central nervous system (CNS), have become the focus of investigations into the sites of action for alcohol and anesthetics. They belong to the Cys-loop superfamily of ligand-gated ion channels, which also includes nicotinic acetylcholine receptor (nAChR), the serotonin 3 receptor (5-HT_3R), and the γ-aminobutyric acid type A receptor ($GABA_A$R). GlyRs mediate inhibitory neurotransmission in the CNS through their expression in the brainstem and spinal cord (Legendre et al., 2001) as well as in areas of the amygdala, cortex, hippocampus, striatum, ventral tegmental area (Jonsson et al., 2009; Baer et al., 2009), and cerebellum (Takahashi et al., 1992).

Functional GlyRs consist of five homologous protein subunits positioned around a central chloride channel, with the glycine agonist binding site located extracellularly at the subunit interface where pairs of oppositely charged residues promote glycine binding (Grudzinska et al., 2005). Structurally, each subunit is composed of an extracellular N-terminal domain, a transmembrane domain with four alpha helical segments (TM1, TM2, TM3, and TM4), an intracellular loop between TM3 and TM4, and an extracellular C-terminal domain. A single β and four α GlyR subunits (1-4) have been identified

(Grenningloh et al., 1990; Harvey et al., 2000; Harvey et al., 2004), which can assemble to form functional homomeric α receptors or heteromeric αβ receptors (Lynch, 2004).

The GlyR is a common site of action for alcohols and anesthetics (Mihic et al. 1997; Mascia et al., 2000; Beckstead et al., 2001; Borghese et al., 2012), and both drugs positively modulate GlyR function (Harrison et al., 1993; Mascia et al., 1996). Studies using site-directed mutagenesis and alkyl thiol reagents have identified critical amino acid residues for alcohol and anesthetic action in TM1-4 of the GlyR and suggest these residues may contribute to water-filled alcohol/anesthetic-binding cavity (Mascia et al., 2000; Jenkins et al, 2001; Lobo et al., 2004a; Lobo et al., 2006). In addition, studies using cysteine mutagenesis and cross-linking agents have shown that A288 in TM3 is able to form cross-links with each of the critical residues for alcohol and anesthetic action in TM1 (I229) (Lobo et al., 2008) (Figure 2.1) , TM2 (S267) (Lobo et al., 2004b), and TM4 (Y406, W407, I409, and Y410) (McCracken et al., 2010). Moreover, cross-linking of A288 in TM3 with 267 in TM2 has been shown to significantly reduce alcohol or volatile anesthetic potentiation of GlyR function (Lobo et al., 2004b), providing further evidence that S267 and A288 together likely participate in an alcohol/anesthetic binding cavity. However in the absence of a high resolution GlyR crystal structure, the specific orientation of these critical residues, particularly A288 in TM3, remains controversial.

Initial medium to high resolution information from related ligand-gated ion channel motifs, including the acetylcholine binding protein (AChBP) (Brecj et al., 2001; Celie et al., 2004) and the *Torpedo* nAChR (Unwin, 2005), provided useful structural insight into GlyR structure that was used to construct early homology models, which

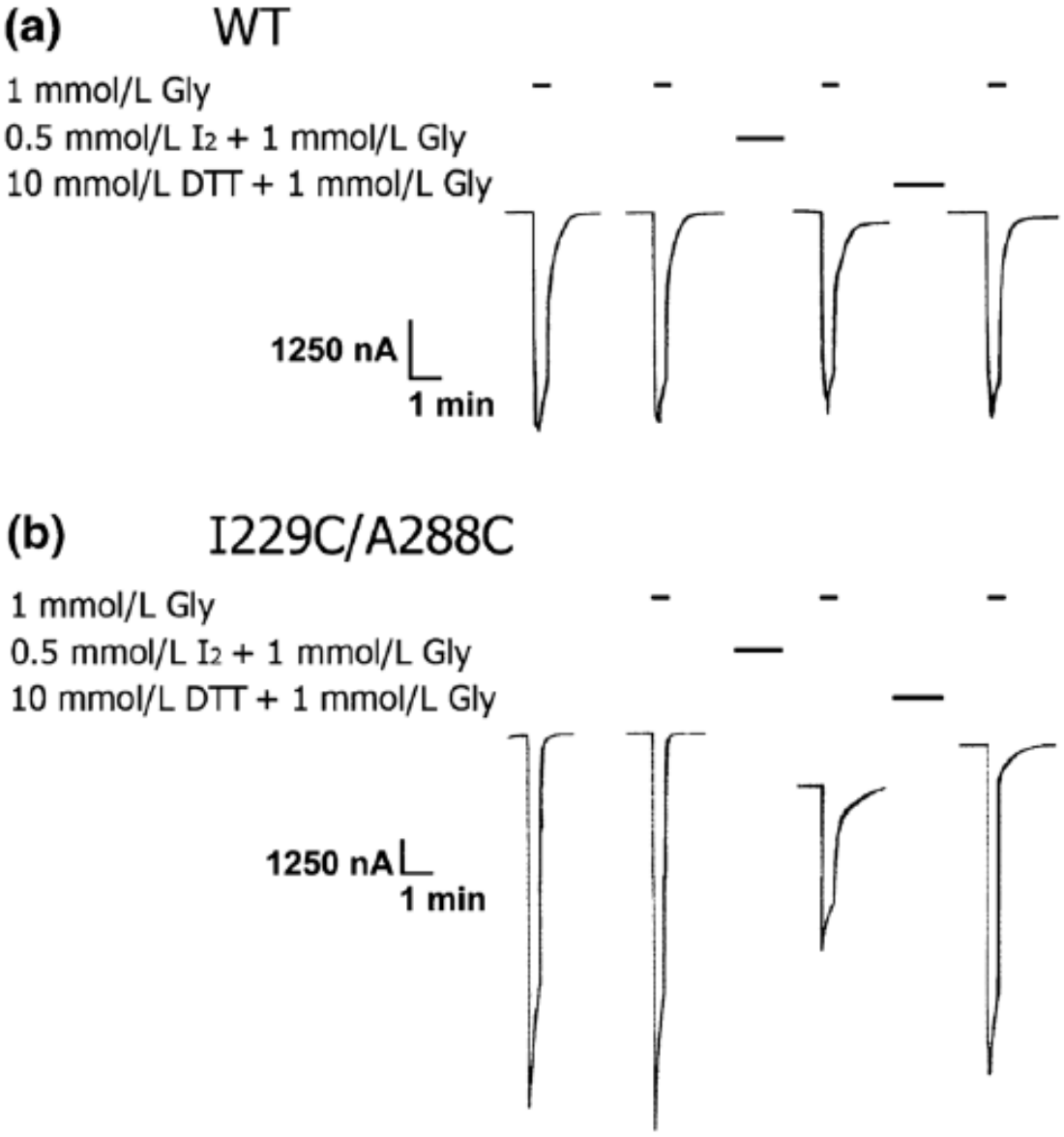

Figure 2.1. Effect of Oxidation with iodine and reduction with DTT on GlyRs. A) WT representative tracing demonstrating that glycine response is altered following the application of 0.5 mM iodine or 10 mM DTT. B) The glycine response of the I229C/A288C double mutant is markedly reduced following oxidation by iodine. However, the effect is reduced by DTT (Adapted from Lobo et al., 2008).

predicted that critical amino acids in all four GlyR TM segments faced into a water-filled alcohol/anesthetic binding cavity within an intra-subunit alpha helical bundle (Bertacini et al., 2005; Ernst et al., 2005; Young et al., 2008). This intra-subunit prediction is consistent, at least in part, with the cross-linking findings from the GlyR demonstrating the ability of A288 in TM3 to form cross-links with each of the critical alcohol/anesthetic residues in the other TM segments. More recently, the x-ray structures have been solved for two prokaryotic homologues, Erwinia chrysanthemi (ELIC) and Gloebacter violaceus (GLIC) (Hilf and Dutzler, 2008; Hilf and Dutlzer, 2009; Bocquet et al., 2009), and the invertebrate Caenorhabditis elegans glutamate-gated chloride channel (GluCl) (Hibbs and Gouaux, 2011). While x-ray structures of general anesthetics bound to the prokaryotic homologue GLIC support binding and modulation at an intra-subunit cavity (Nury et al., 2009), functional and structural studies in GLIC using ethanol and bromo-ethanol instead propose an alternative inter-subunit hypothesis whereby alcohol action is primarily occurring at the interface of two adjacent subunits (Sauguet et al., 2013). A third "multisite" hypothesis has also been generated based on mutagenesis studies in the GLIC and GluCl homologues, molecular simulations, and newer homology models. The multisite hypothesis posits that alcohols and anesthetics may, in fact, act at both inter-subunit and intra-subunit sites within these protein receptors (Howard et al. 2011; Murail et al., 2012; Yoluk et al., 2013). Therefore, the aim of the present study was to investigate whether 1229 in TM1 and A288 in TM3 are participating in a common alcohol/anesthetic binding cavity, and to determine whether the cavity is within a GlyR subunit or between adjacent subunits.

MATERIALS AND METHODS

Materials

All chemical reagents and buffer constituents were purchased from Sigma-Aldrich (St. Louis, MO), and were prepared immediately before use. Adult female *Xenopus laevis* frogs were purchased from Nasco (Ft. Atkinson, WI).

Site-directed Mutagenesis

A QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to make point mutations in the human glycine receptor α_1 subunit (subcloned in the pBKCMV N/B-200 vector). Specifically, the I229C and A288C single mutants were constructed. Additionally, the I229C/A288C, and I229C/C290S double mutants and the I229C/A288C/C290S triple mutant were engineered. All point mutations were verified by DNA sequencing in the core facility at the University of Texas at Austin.

Oocyte Isolation and cDNA Injection

Portions of ovary were surgically extracted from adult female *Xenopus laevis* frogs. Mature oocytes were manually isolated, treated in 0.5 mg/ml collagenase (type IA), and subsequently injected into the nucleus with 30 nl of nuclease-free water and cDNA (1.5 ng/30 nl) encoding either wild-type or mutant human α_1 glycine receptors. Injected oocytes were incubated at 13°C in sterile modified Barth's solution (MBS) (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 19 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM

$Ca(NO_3)_2$, 0.91 mM $CaCl_2$, 10,000 units/liter penicillin, 50 mg/liter gentamicin, 90 mg/liter theophylline, 220 mg/liter sodium pyruvate, pH 7.5) for 1-7 days.

Electrophysiological Recording

Two-electrode voltage clamp electrophysiology was used to measure glycine-induced current from oocytes 1-7 days following injection with cDNAs. Oocytes were place in a rectangular chamber (~ 100 μl) and continuously perfused at a rate of 2 ml/min with MBS at 13°C. A -70 mV holding potential was achieved using to two glass electrodes containing 3 M KCl and a Warner Instrument oocyte clamp (Hamden, CT). All solutions were prepared immediately before use and applied by bath perfusion.

Alcohol or Volatile anesthetic potentiation. A glycine EC_{5-10} (the concentration that produced 5-10% of the maximal response) was determined for each oocyte after application of the maximal glycine concentration and served as the test glycine concentration. All test glycine concentrations were applied for 30 s and were followed by a 5 min washout. Oocytes received two consecutive applications of the test glycine concentration to ensure that responses were stable. Alcohols or anesthetics were pre-applied for 1 min alone and then co-applied with glycine for 30 s. All drug applications were immediately followed by a 10 min washout. The test glycine concentration was applied again, and the percent potentiation of the glycine-induced current by the alcohol or anesthetic was calculated for each oocyte. 10 μM $HgCl_2$ was applied for 1 min in the presence of 100 mM glycine (maximal) and followed by a 15 min washout. Afterward,

the maximal glycine concentration was re-applied and the test concentration (EC_{5-10}) was recalculated. The potentiation of glycine-induced current by the alcohol or anesthetic was then measured again as described above. This protocol was used to test 22 mM butanol and 0.6 mM isoflurane, which corresponds to approximately two times the minimal alveolar concentration (MAC) for these drugs.

Oxidation with H_2O_2 and Reduction with DTT. The maximal glycine concentration (100 mM) was applied for 15-20 s and followed by a 15 min washout. This was repeated and the second glycine response served at the test glycine response for this experiment. 0.5% H_2O_2 was then applied in the presence of maximal glycine (100 mM) for 1 min. and then washed out for 15 min. The oocytes were unclamped during application and washout of the oxidizing agent to maintain health, but remained impaled by the electrodes as previously described in Lobo et al. 2008 and McCracken et al. 2010. After re-clamping, maximal glycine was re-applied and the responses were compared to the initial glycine test response prior to H_2O_2 application. Maximal glycine was applied a second time and served as the new test glycine response. 10 mM dithiothreitol (DTT) was applied for 3 min while oocytes were unclamped as described above for the cross-linking agents and washed out for 15 min and re-clamped. Maximal glycine was re-applied and the responses were compared to the second glycine test response following H_2O_2 application.

Data Analysis. For each receptor tested in the experiments using $HgCl_2$, repeated-measures *t*-tests were used to detect differences before and after cross-linking (i.e. pre-$HgCl_2$ vs post-$HgCl_2$) conditions. For the additional cross-linking experiments using the

oxidizing agent H_2O_2 and reducing agent DTT, a one-way ANOVA with Tukey's post-test was used to detect significant differences between conditions (pre- H_2O_2 vs post-H_2O_2 vs post-DTT). GraphPad PRISM software (San Diego, CA) was used for all analyses, and statistical significance was determined at $p < 0.05$.

Protein Extraction

Individual oocytes were manually isolated and injected with 1.5 ng/nl cDNA as described above. Injected oocytes were incubated for 5-7 days at 13°C. Groups of 25 oocytes were pooled for each condition. Those included in the cross-linked condition were treated with 0.5 % H_2O_2 by bath perfusion as described in McCracken et al. 2010 and as described for the $HgCl_2$ application above in the electrophysiology experiments. Following a 15 min washout, oocytes were homogenized in 1 mL of Wash Buffer (0.1M EDTA, pH 7.5; 0.1 M EGTA, pH 7.5; 2M NaCl; 0.1M NaH_2PO_4, pH 7.5) with protease inhibitors (5 mM benzamidine; 15 mM iodoacetamide), and then centrifuged at 4°C for 30 min at 13500 rpm. The supernatant was removed, and 250 μL of Extraction Buffer (wash buffer described above + 2% Triton + 5 mM benzamidine + 15 mM iodoacetamide) was added. The pellet was resuspended, rotated at 4°C for 2 hrs, and then centrifuged at 4°C for 30 min at 13500 rpm. The supernatant was removed and saved as the protein extract. Similar protein extract protocols using *Xenopus* oocytes have been described previously by Bali et al. (2009).

Immunoblotting

Equal amounts of soluble proteins (unless otherwise noted) extracted from oocytes were resolved by SDS-PAGE and electrotransferred to polyvinylidene fluoride membrane in a buffer containing 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, and 20% (v/v) methanol for 1 h at 18 V. Membranes were briefly rinsed in a buffer containing 10 mM Tris HCl (pH 8.0), 150 mM NaCl and 0.01% (v/v) Tween-20 (TBS-T), and then incubated at room temperature for 1 h in TBS-T containing 5% (w/v) skimmed milk powder to block non-specific binding of antibodies. Incubation of membranes with specific primary antibody for GlyR α1 (AbCam, Cambridge, England) was performed in TBS-T containing 1% skimmed milk and 1% BSA powders (w/v) for 2 h at room temperature, followed by three 10 min washes with TBS-T alone. Membranes were then incubated for 1 h at room temperature with an appropriate secondary antibody conjugated to horseradish peroxidase (HRP), diluted in TBS-T /1% (w/v) skimmed milk and BSA powders, followed by three 5 min washes with TBS-T. After the final wash, blots were immediately developed by applying the Enhanced Chemi-Luminescence (ECL) reagent (Pierce Chemical Co., Rockford, IL) for 2 min, and then a Kodak Image Station 2000MM (Eastman Kodak, Rochester, NY) was used to acquire images.

Protein Identification by Mass Spectrometry

Gel bands of interest were excised and washed 3 times then digested in-gel with modified porcine trypsin protease (Promega, Fitchburg, WI). The digested tryptic peptides were desalted using a Zip-tip C18 (Millipore, Billerica, MA). Peptides were

eluted from the Zip-tip with 0.5μL of matrix solution (α-cyano-4-hydroxycinnamic acid, 5 mg/mL in 50% acetonitrile, 0.1% trifluoroacetic acid, 25mM ammonium bicarbonate) and spotted on a MALDI plate.

Mass Spectrometry. MALDI-TOF MS and TOF/TOF tandem MS/MS were performed on AB SCIEX TOF/TOF™ 5800 System (AB SCIEX). MALDI-TOF mass spectra were acquired in reflectron positive ion mode, averaging 4000 laser shots per spectrum. TOF/TOF tandem MS fragmentation spectra were acquired for each sample, averaging 4000 laser shots per fragmentation spectrum on each of the 7-10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions).

Database search. Both the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix Science, Boston, MA) to search the Swiss-Prot database. Searches were performed without constraining protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and with one missed cleavage also allowed in the search parameters. Protein identification in the mass spectra was performed by Applied Biomics (Hayward, CA).

Quantification of Band Ratios

ImageJ64 was used to process and quantify α1 GlyR-labeled band intensity at approximately 100 kDa and 50 kDa, which corresponded to the loci in which monomeric and dimeric GlyR subunits were identified by MS. These band intensities were then

calculated and reported as direct ratios (100:50 kDa), and statistically significant differences in band ratio between the uncross-linked and cross-linked conditions were measured for the representative TM1-3 mutant and the wild-type using repeated-measures *t*-tests, $p < 0.05$.

RESULTS

Previous studies using cysteine substitution and alkyl thiol reagents have shown that I229 in TM1 and A288 in TM3 are water-accessible and play critical role in anesthetic action (Jenkins et al., 2001; Lobo et al., 2004a; Lobo et al., 2004b), and initial biochemical cross-linking studies showed evidence of cross-link formation and suggested I229 and A288 likely participate in a common alcohol and volatile anesthetic binding cavity. However, it remains uncertain whether the I229C/A288C cross-links are formed between residues within the same subunit (intra-subunit) or between subunits (inter-subunit), and whether these residues are participating in common alcohol-binding cavity within or between GlyR subunits. Therefore, the purpose of the present study was to examine these possibilities (i.e. intra-subunit vs inter-subunit cross-linking) using cysteine mutagenesis, Xenopus oocyte electrophysiology, and immunoblotting with a GlyR-specific antibody.

The I229C/A288C double mutant and I229C/A288C/C290S triple mutant previously shown to form cross-links in Lobo et al. (2008) as well as the corresponding single mutants were each expressed in oocytes. Glycine-induced current was measured, and the alcohol and volatile anesthetic effects were compared before and after cross-linking with $HgCl_2$, which reacts with accessible pairs of cysteines to form intermolecular S-Hg-S dimers when the residues are in proximity to one another and located on opposing faces of adjacent helices (Struthers et al., 2000; Soskine et al., 2002). The glycine sensitivity of the cysteine mutants was reported (Lobo et al., 2008), and although

the mutations produced some degree of altered glycine sensitivity (Table1), all of the mutants formed functional GlyRs.

First, we examined whether the cross-linking of I229C in TM1 with A288C in TM3 alters alcohol enhancement of GlyR function. Specifically, the potentiation of submaximal (EC_{5-10}) glycine-induced current by 22 mM butanol was compared before and after the application of the cross-linking agent $HgCl_2$. Similar to Lobo et al. (2008), agonist-induced rotation of the TM domain appears to be necessary for the formation of cross-links in the I229C/A288C mutant, so 10 μM $HgCl_2$ was applied in the presence of 100 mM glycine (maximal) for these experiments. We found butanol potentiation was nearly abolished in both the I229C/A288C double mutant and I229C/A288C/C290S triple mutant following the application of $HgCl_2$, but there was no significant effect on butanol potentiation in the wild-type or single mutants (Figure 2.1).

Next, we investigated whether the cross-linking of I229C in TM1 with A288C in TM3 alters volatile anesthetic enhancement of GlyR function. Specifically, we measured the potentiation of submaximal (EC_{5-10}) glycine current by 0.6 mM (2xMAC) isoflurane before and after the application of $HgCl_2$. As described above for the butanol experiment, $HgCl_2$ was applied in the presence of maximal glycine. There was no effect of $HgCl_2$ on the wild-type or single mutants. However, isoflurane potentiation was significantly decreased in both the I229C/A288C double mutant and I229C/A288C/C290S triple mutant (Figure 2.2).

In order to verify the results of $HgCl_2$ cross-linking by an alternative method in preparation for the immunoblotting experiments, we tested the ability of the oxidizing

Receptor	Glycine EC_{50} (μmol/L)
WT	180 ± 56
I229C	110 ± 7.0
A288C	1800 ± 190
I229C/A288C	180 ± 37
I229C/A288C/C290S	81 ± 45

Table 1
Glycine EC_{50} values for wild-type and mutant TM1-3 glycine receptors. All values represent mean ± SEM from 4-11 oocytes. Adapted from Lobo et al., 2008.

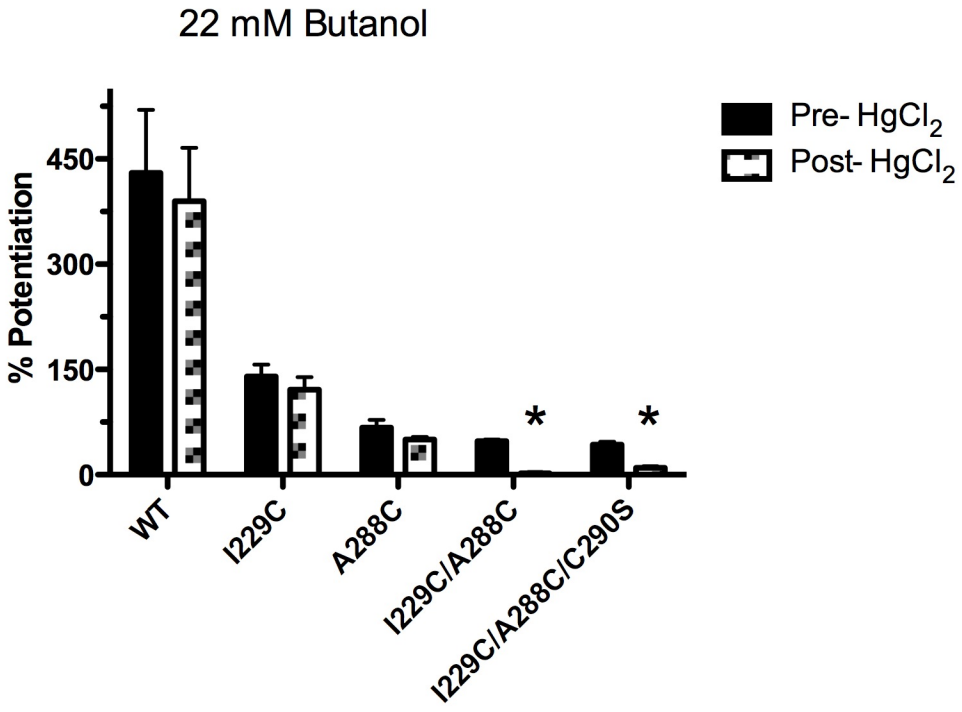

Figure 2.2. Alcohol potentiation of EC_{5-10} glycine response in mutant TM1-3 GlyRs. As described in Lobo et al. (2008), 10 μM $HgCl_2$ was applied in the presence of 100 mM glycine (i.e. the activated/desensitized state) to the I229C/A288C double mutant and the I229C/A288C/C290S triple mutant (as well as single mutant controls) and produced evidence of cross-linking. The effect of 22 mM butanol was compared before and after 10 μM $HgCl_2$ was applied in the presence of glycine to the mutants and the wild-type. All values represent mean ± SEM from 4-5 oocytes. Repeated-measures t-tests were used to detect differences between the Pre-$HgCl_2$ and Post-$HgCl_2$ conditions for each receptor. *$p<0.05$

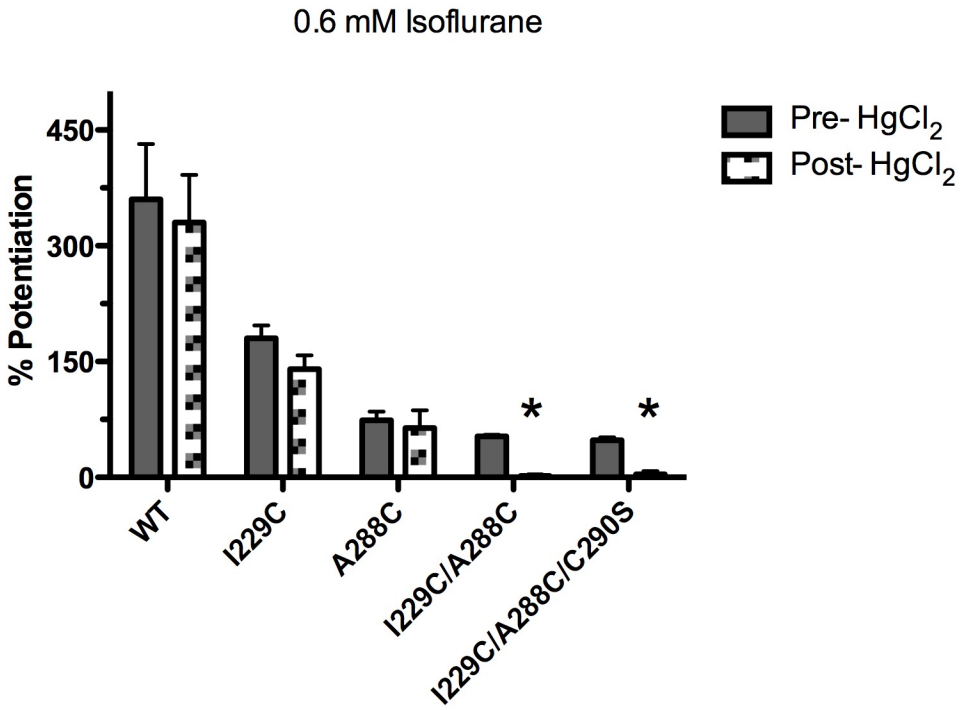

Figure 2.3. Anesthetic potentiation of $EC_{5\text{-}10}$ glycine response in mutant TM1-3 GlyRs. As described in Lobo et al. (2008), 10 μM $HgCl_2$ was applied in the presence of 100 mM glycine (i.e. the activated/desensitized state) to the I229C/A288C double mutant and the I229C/A288C/C290S triple mutant (as well as corresponding single mutant controls) and produced evidence of cross-linking. The effect of 0.6 mM isoflurane was similarly tested on the mutants and the wild-type before and after 10 μM $HgCl_2$ was applied in the presence of glycine. All values represent mean ± SEM from 4-5 oocytes. Repeated-measures t-tests were used to detect differences between the Pre-$HgCl_2$ and Post-$HgCl_2$ conditions for each receptor. *$p<0.05$

agent, H_2O_2, to induce disulfide bond formation in the I229C/A288C double mutant that showed evidence of cross-linking. We also tested the I229C/C290S double and I229C/A288C/C290S triple mutants to help address the possibility that I229C in TM1 may be cross-linking with the endogenous cysteine at position 290, instead of A288. In accordance with the $HgCl_2$ experiments and iodine studies previously conducted by Lobo et al. (2008), cross-linking was again observed for the I229C/A288C double and the I229C/A288C/C290S triple mutants following the application of 0.5% H_2O_2, resulting in significantly decreased glycine-induced current. The effect was reversed by reduction with 10 mM DTT. However, no cross-linking was observed for the wild-type, I229C single mutant, or I229C/C290S double mutant (Figure 2.4). The I229C/A288C/C290S triple mutant appears to form cross-links in an almost identical manner to that of the I229C/A288C double mutant, suggesting that the endogenous cysteine at position 290 is not responsible for the effects observed and the cross-links are forming between I229 in TM1 and A288 in TM3. Therefore, the I229C/A288C/C290S triple mutant was not included in the remainder of this study, and we focused on the I229C/A288C double mutant in order to elucidate whether the cross-links formed between I229 and A288 are occurring within the same subunit (intra-subunit) or between subunits (inter-subunit), and whether these residues are participating in a common alcohol-binding cavity within or between GlyR subunits.

To assess these possibilities further, we extracted protein from oocytes injected with wild-type GlyRs or the I229C/A288C double mutant from above and then used immunoblotting with a GlyR-specific antibody (Figure 2.5A and B). We subsequently

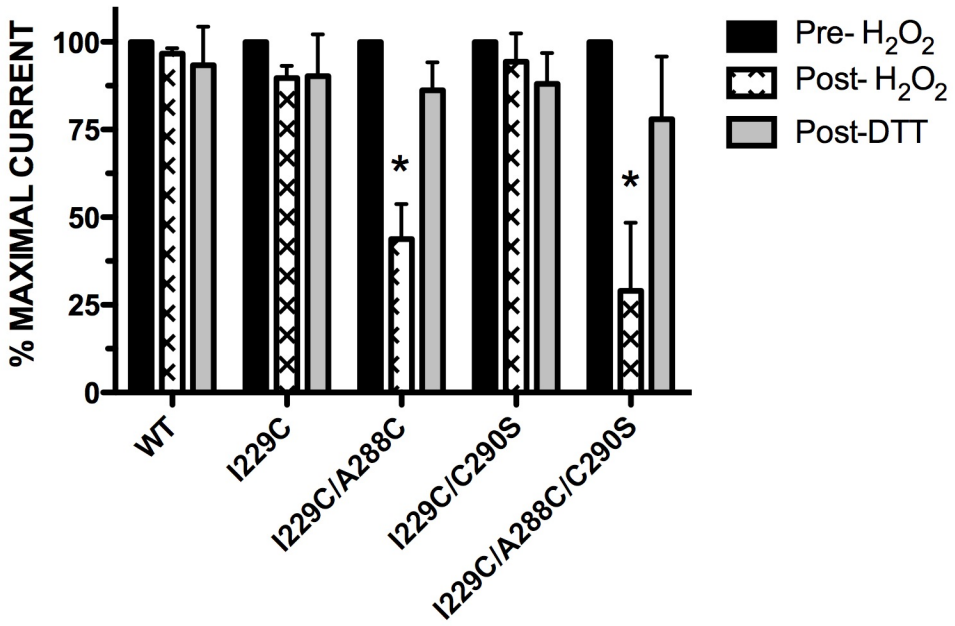

Figure 2.4. Effects of oxidation with H_2O_2 and reduction with DTT on GlyRs. 0.5% H_2O_2 was applied in the presence of 100 mM glycine (i.e. the activated/desensitized state), which produced evidence of cross-linking in the I299C/A288C and I229C/A288C/C290S mutants only. This effect was reversed by 10 mM DTT. All values represent mean ± SEM from 4-5 oocytes. One-way ANOVA with Tukey's post-test was used to detect differences between the Pre- H_2O_2, Post- H_2O_2, and Post-DTT conditions for each receptor. *$p<0.05$

verified protein identity by mass spectrometry (MALDI-TOF/TOF) (Table 2). The differences in 100:50 kDa band ratios were compared between uncross-linked and cross-linked conditions for the wild-type and I229C/A288C double mutant. Wild-type GlyR subunits measure about 50 kDa under ordinary conditions. Therefore, we hypothesized that if cross-linking occurred between two adjacent subunits, then we would observe an increase in the presence of GlyR-labeled 100 kDa band, and an increase in the ratio of 100:50 kDa bands. Alternatively, we expected no change for cross-linking within a subunit. For the I229C/288C mutant, we found a significant increase in the 100:50 kDa band ratio (Figure 2.6), which suggests that TM1-3 may participate in an alcohol/anesthetic binding cavity between GlyR subunits. There was not a significant difference in the 100:50 kDa band ratio between the uncross-linked or cross-linked condition for the wild-type.

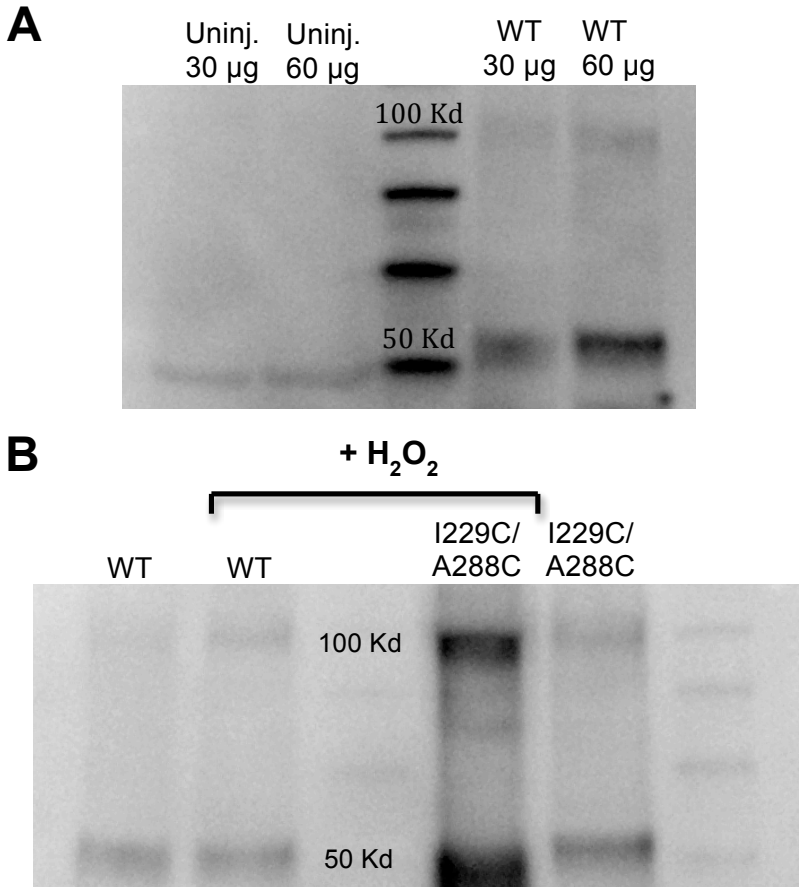

Figure 2.5. Immunoblot for WT and mutant TM1-3 GlyRs. A) Immunblotting reveals a dominant 50 kD band, the anticipated molecular weight of a GlyR subunit, with increasing concentrations of wild-type GlyR protein, unlike in the lanes loaded with samples comprised of uninjected (uninj) oocytes, which only showed faint evidence of non-specific labeling at lower molecular weight. B) 100 kD and 50 kD α1 GlyR-labeled bands in the wild-type and I229C/A288C mutant in the uncross-linked vs cross-linked ($+H_2O_2$) conditions.

	Molecular Weight (kDa)	Protein Score C.I. %
GLRA1	100-105	59
GLRA1	100	59
GLRA1	50-55	87
GLRA1	48-50	78

Table 2
Protein Identification of Wild-Type GlyR. All values are from a representative uncross-linked wild-type sample determined by MALDI-TOF/TOF.

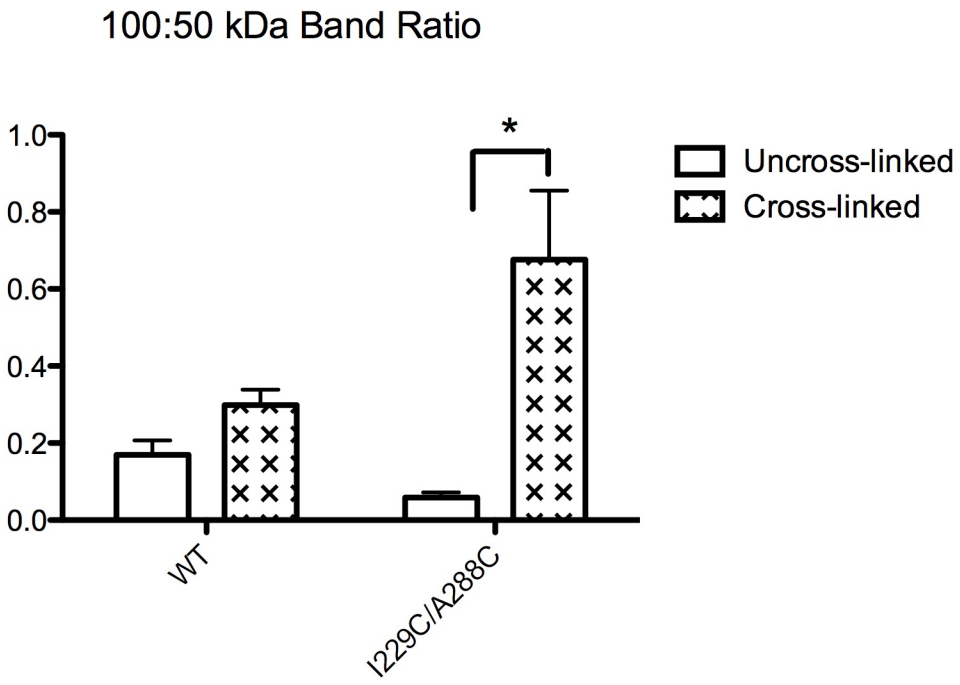

Figure 2.6. Quantification of Band Ratios for WT and TM1-3 mutant GlyRs. The100:50 kDa band ratio increased for the I229C/A288C double mutant when cross-linked, however there was no significant increase for the wild-type. Immunoblot images were processed using ImageJ64 software, and the band intensity was taken and reported as a direct ratio. 0.5% H_2O_2 was applied by bath perfusion as previously described (McCracken et al., 2010). Independent t-tests were used to detect differences between the Uncross-linked and Cross-linked conditions for each receptor. *$p<0.05$

DISCUSSION

Our findings suggest that the formation of a cross-link between residue 288 in TM3 and residue I229 in TM1 reduces the ability of alcohol and volatile anesthetics to bind and produce their effects at an inter-subunit cavity in the GlyR. These results are in accordance with similar studies in the $GABA_AR$. For example, Bali et al. (2009) suggested that the homologous residue to I229 in TM1 of the $GABA_AR$ is oriented toward the subunit interface such that it is able to form cross-links with TM3 of an adjacent subunit, and photoaffinity labeling studies have suggested that multiple classes of general anesthetics may act at this inter-subunit site (Zhong et al., 2008; Stewart et al., 2008; Li et al., 2010). Moreover, crystallographic studies using an ethanol-sensitized GLIC mutant co-crystallized with ethanol, bromoethanol and bromoform provide additional support for an inter-subunit binding cavity (Sauguet et al., 2013).

These results differ, at least in part, from the recent x-ray structure of the volatile anesthetic desflurane bound at an intra-subunit cavity in the bacterial homologue GLIC (Nury et al., 2011) and suggest that anesthetic interaction with GLIC may involve more than a simple binding cavity either between or within subunits. Similarly, Borghese et al. (2014) reported evidence for an intra-subunit alcohol/anesthetic binding cavity in the $GABA_AR$ β_2 subunit using biochemical cross-linking and alkyl thiol labeling techniques, which specifically revealed that residue N265 in TM2 (homologous to S267 in TM2 of the α_1 GlyR) could form cross-links with other β_2 residues in both TM1 and TM3 of the same subunit. This appears to be consistent with earlier studies in the α_1 GlyR in which

the formation of a cross-link between A288 in TM3 and 267 in TM2 significantly reduced alcohol or volatile anesthetic effects (Lobo et al., 2004b). However, in accordance with our findings here and the work mentioned above by Bali et al. (2009), Borghese et al. (2014) also found evidence for inter-subunit cross-linking between the homologous TM1 residue in the $GABA_AR$ α_1 subunit (L232) and the TM3 residue in β_2, but one helical turn below the site homologous to A288 in the GlyR. This further suggests that, in addition to GLIC, alcohol and anesthetic interaction with these critical residues in the mammalian ion channels also involves additional complexity beyond exclusive binding either at inter-subunit or intra-subunit site.

Additionally, the results from our oxidation experiment in the present study corroborate earlier cross-linking studies in the GlyR that initially investigated the proximity and orientation of residue A288 in TM3 with residues in TM1, particularly the critical alcohol and anesthetic residue at I229. Previously, Lobo et al. (2008) used iodine and $HgCl_2$ to independently test for cross-linking in a number of GlyR TM1 and TM3 double cysteine mutants. Of note, evidence of cross-linking was reported in the I229C/A288C and I229C/A288C/C290S double and triple mutants, which was reversible with DTT following application of either $HgCl_2$ or iodine. This is in agreement with our present findings using H_2O_2 and $HgCl_2$, which we validated with immunoblotting. Based on earlier homology modeling used in Lobo et al. (2008), it was suggested that, perhaps, cross-linking was occurring between TM1 and TM3 of the same subunit. At the time, the 4Å resolution structure of the *Torpedo* nAChR was commonly used as a template to model members of the Cys-loop family of ion channels, including the GlyR. However,

the lack of some conserved residues created sequence alignment challenges between the nAChR and GlyR, especially in the TM2-3 loop and TM3 region, which resulted in added uncertainty about the orientation and proximity of residues in TM3. The emergence of x-ray structures for the prokaryotic homologues ELIC and GLIC as well as the invertebrate GluCl have since provided greater insight and helped to clarify some these alignment discrepancies. Nevertheless, the combination of the present study and Lobo et al. (2008) demonstrate that the earlier homology models based on the *Torpedo* nAChR still proved valuable in helping to identify critical residues for further experimental testing in heterologous expression systems and yielded useful predictions and hypotheses about the location of putative alcohol/anesthetic binding pockets with the GlyR.

When considering the accumulating experimental evidence from studies in the GlyR as well as studies of the closely related $GABA_AR$, it becomes increasingly difficult to explain the inter-subunit cross-linking of TM3 with TM1 in an adjacent subunit and intra-subunit cross-linking with TM2 or TM4 of the same subunit (Lobo et al., 2004b; Bali et al., 2009; McCracken et al., 2010; Borghese et al., 2014) without a substantial degree of flexibility or motion, especially in the upper portion of TM3. Figure 2.7 (adapted from Bertaccini et al., 2010) illustrates the predicted orientation of I229 in TM1 and A288 in TM3 of adjacent GlyR subunits with an anesthetic molecule drawn at the subunit cleft, and also notes the proximity of TM4 residues which have been previously shown to cross-link with A288 in TM3. Based on hGlyR-α1 TM NMR spectroscopy studies, Mowrey et al. (2013) reported a highly dynamic segment in the TM region

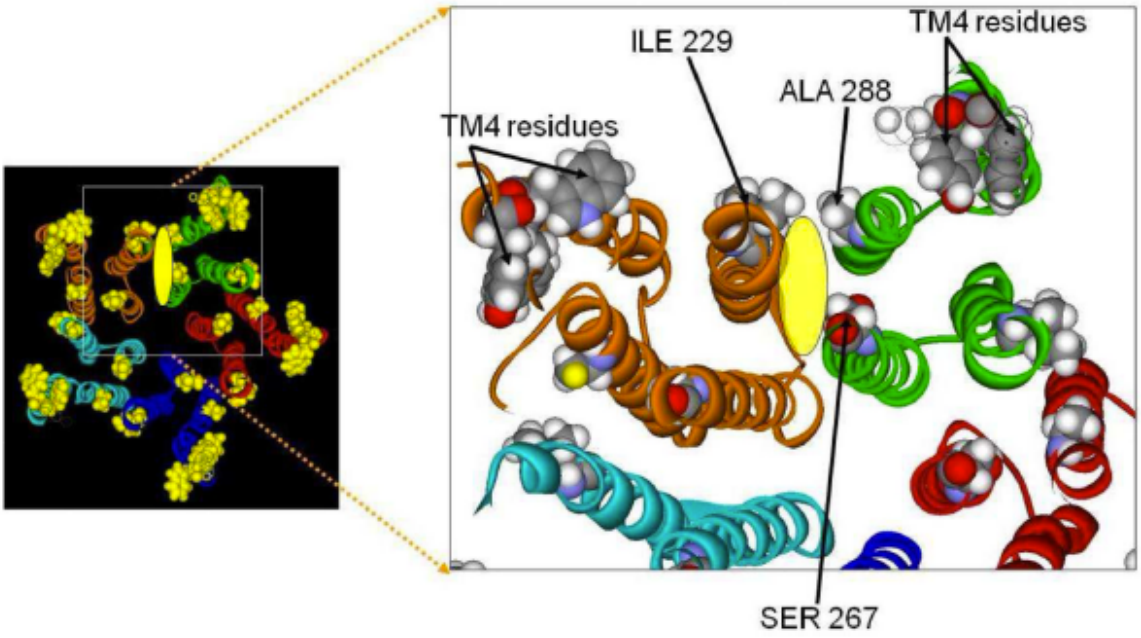

Figure 2.7 Overhead view of critical GlyR TM sites for anesthetic action. Adjacent subunits are distinguished by color, gold and green, etc. A hypothetical anesthetic molecule is drawn in the proposed inter-subunit site at the subunit cleft. This homology model is based on the prokaryotic homologue GLIC (adapted from Bertaccini et al., 2010).

encompassing S267 in TM2 through approximately A288 in TM3. This may help to explain the ability of A288 to cross-link with I229 in TM1 across the subunit cleft, and alternatively, also potentially cross-link in the opposite direction with 267 in TM2 or the residues in TM4 (Y406, W407, I409, Y410). This seems plausible given that a number of these mutants, including I229 in TM1 and Y406 and I409 in TM4, require the co-application of glycine in the presence of the cross-linking/oxidizing agent. It has been suggested that the application of agonist creates a wringing motion in the extracellular domain due to ligand binding and may result in downstream rotation in the TM segments (Unwin, 2005). Another, similar example of motion has been described in the GluCl-ivermectin model (Yoluk et al., 2013).

In summary, we demonstrate that cross-linking between I229 in TM1 and A288 in TM3 reduces the ability of alcohol or anesthetic to enhance GlyR function. In addition, we provide evidence using immunoblotting that these cross-links are formed between adjacent subunits, suggesting that TM1 and TM3 likely participate in an inter-subunit alcohol/anesthetic binding cavity in the GlyR.

CHAPTER III: Evidence for the Participation of Transmembrane Segments 3 and 4 in a Common Intra-subunit Alcohol and Volatile Anesthetic Binding Cavity in the Glycine Receptor

INTRODUCTION

GlyRs are members of the Cys-loop family of ligand-gated ion channels, which includes the nicotinic acetylcholine receptor (nAChR), the serotonin 3 receptor (5-HT_3R), and γ-aminobutyric acid type A ($GABA_A$R). GlyRs not only mediate the majority of inhibitory neurotransmission in the brain stem and spinal cord (Legendre, 2001), but they are also expressed in other brain regions including the olfactory, hippocampus, nucleus accumbens (van den Pol and Gorces, 1988; Fatima-Shad and Barry, 1993; Molander and Soderpalm, 2005; Baer et al., 2009; Jonsson et al., 2012) and cerebellum (Takahashi et al., 1992). Although a GlyR crystal structure has not yet been solved, medium to high resolution structures exist for related ligand-gated ion channel motifs, including the acetylcholine binding protein (AChBP) (Brecj et al., 2001; Celie et al., 2004) and the *Torpedo* nAChR (Unwin, 2005). Additionally, the x-ray structures have been solved for two prokaryotic homologues, Erwinia chrysanthemi (ELIC) and Gloebacter violaceus (GLIC) (Hilf and Dutzler, 2008; Hilf and Dutlzer, 2009; Bocquet et al., 2009), and the invertebrate Caenorhabditis elegans glutamate-gated chloride channel (GluCl) (Hibbs and Gouaux, 2011). Most recently, Mowrey et al. (2013) usednuclear magnetic resonance (NMR) spectroscopy and electron microroscopy to produce specific structural information from the expressed hGlyR-α1 TM domain. This lower resolution structural

information, as well as these high-resolution x-ray structures, combined with experimental data from studies in heterologous expression systems, has provided insight into GlyR structure.

Functional GlyRs receptors are composed of five homologous subunits positioned around a central chloride channel, with the structure of each subunit consisting of an extracellular N-terminal domain, a transmembrane domain with four alpha helical segments (TM1, TM2, TM3, and TM4), an intracellular loop between TM3 and TM4, and an extracellular C-terminal domain. Four GlyR α subunits (1-4) and a single β subunit have been identified (Grenningloh et al., 1990; Harvey et al., 2000; Harvey et al., 2004). There is 80-90% sequence identity among the α subunits and approximately 50% identity among the β subunit and the α subunits, which can assemble to form either homomeric α receptors or heteromeric αβ receptors (Lynch, 2004).

It is well established that alcohols and volatile anesthetics likely share common sites of action in the GlyR (Mihic et al., 1997; Beckstead et al., 2001) and serve as positive allosteric modulators of GlyR function (Harrison et al., 1993; Mascia et al., 1996). Moreover, critical amino acid residues for alcohol and anesthetic enhancement of the α1 GlyR have been identified in TM1-3 (I229, S267, and A288 in TM1, 2, and 3, respectively) (Mihic et al., 1997; Lobo et al., 2004a). Although the role of TM4 in alcohol and anesthetic action at the GlyR has not been as extensively examined as TM1-3, studies using site-directed mutagenesis and alkyl thiol reagents suggest that TM4 residues, along with the implicated residues in TM1-3, may face into a water-filled,

alcohol/anesthetic-binding cavity within the TM domain of each subunit (i.e. an intra-subunit binding pocket) (Mascia et al., 2000; Jenkins et al., 2001; Lobo et al., 2004a; Lobo et al., 2006).

In accordance, early homology modeling based on the nAChR predicted that A288 of TM3 could face into an intra-subunit helical bundle (Ernst et al., 2005; Young et al., 2008), which is consistent with experimental biochemical cross-linking data demonstrating the ability of A288 in TM3 to form cross-links with I229 in TM1 and/or S267 in TM2 (Lobo et al., 2004b; Lobo et al., 2008). More recent studies using cysteine mutagenesis and oxidizing agents have begun to investigate the proximity of A288 in TM3 to residues in TM4 and provide evidence that Y406, W407, I409, and Y410 in TM4 are able to form cross-links with A288 in TM3, which supports the assignment of these TM4 residues as intra-subunit facing (McCracken et al., 2010; Bertacini et al., 2010). In addition, recent x-ray structures of anesthetics bound to the bacterial homologue GLIC and photolabeling of $GABA_A$ receptors further support action at an intra-subunit cavity (Nury et al., 2011)

Alternatively, others have proposed the presence of an inter-subunit alcohol and general anesthetic binding pocket at the subunit interface. Specifically, Bali et al. (2009) suggested that the homologous residue to A288 in TM3 of the $GABA_A$ receptor is oriented toward the subunit interface such that it is able to form cross-links with TM1 residues of an adjacent subunit, and photoaffinity labeling studies have suggested that multiple classes of general anesthetics may act at this inter-subunit site (Zhong et al., 2008; Stewart et al., 2008; Li et al., 2010). This inter-subunit hypothesis has been

recently supported by crystallography work paired with functional studies in GLIC, which determined and compared the crystal structures of receptors in the presence and absence of ethanol and bromoethanol (Sauguet et al., 2013).

Lastly, a third "multisite" hypothesis has emerged suggesting that perhaps alcohols and anesthetics may, in fact, bind and act at both intra-subunit and inter-subunit sites within GlyR proteins. This hypothesis has been generated largely from mutagenesis studies in the GLIC and GluCl homologues, molecular simulations, and newer homology modeling (Howard et al. 2011; Murail et al., 2012; Yoluk et al., 2013). Recent cross-linking studies in the mammalian $GABA_A$ receptor also provide additional support for the "multisite" hypothesis, but suggest that the effects may be influenced by receptor type and state as well as differentially affected by alcohols and anesthetics (Borghese et al., 2013).

However in the absence of a crystal structure for either the GlyR or the $GABA_AR$, the orientation of these residues, and ultimately a putative alcohol and volatile anesthetic binding pocket, remains uncertain. Therefore, the goal of the present study was to investigate the possibility that A288 in TM3 participates in cross-linking with intra-subunit facing residues in TM4. To do so, we examined whether cross-linking A288 with each of the intra-subunit facing residues in TM4 (Y406, W407, I409, and Y410) altered alcohol or volatile anesthetic enhancement of α1 GlyR function.

MATERIALS AND METHODS

Materials

All chemical reagents and buffer constituents were purchased from Sigma-Aldrich (St. Louis, MO). Adult female Xenopus laevis frogs were obtained from Nasco (Ft. Atkinson, WI).

Site-directed Mutagenesis

Point mutations in the human glycine receptor α_1 subunit (subcloned in the pBKCMV N/B-200 vector) were achieved using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Specifically, the A288C, Y406C, W407C, I408C, I409C, and Y410C single mutants were constructed. Additionally, the A288C/Y406C, A288C/W407C, A288C/I409C, and A288C/Y410C double mutants were made. All point mutations were verified by DNA sequencing in the core facility at the University of Texas at Austin.

Oocyte Isolation and cDNA Injection

Portions of ovary were surgically extracted from adult female Xenopus laevis frogs, and mature oocytes were manually isolated. Following isolation, ooctytes were treated in 0.5 mg/ml collagenase (type IA) and subsequently injected into the nucleus with 30 nl of nuclease-free water and cDNA (1.5 ng/30 nl) encoding either wild-type or mutant human α_1 glycine receptors. Injected oocytes were incubated at 13°C in sterile modified Barth's solution (MBS) (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 19 mM

HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, 10,000 units/liter penicillin, 50 mg/liter gentamicin, 90 mg/liter theophylline, 220 mg/liter sodium pyruvate, pH 7.5) for 1-7 days.

Electrophysiological Recording

Two-electrode voltage clamp electrophysiology was used to measure glycine-induced current from oocytes 1-7 days following injection with cDNAs. Oocytes were place in a rectangular chamber (~ 100 μl) and continuously perfused at a rate of 2 ml/min with MBS at 13°C. A -70 mV holding potential was achieved using to two glass electrodes containing 3 M KCl and a Warner Instrument oocyte clamp (Hamden, CT). All solutions were prepared immediately before use and applied by bath perfusion.

A glycine EC_{5-10} (the concentration that produced 5-10% of the maximal response) was determined for each oocyte after application of the maximal glycine concentration and served as the test glycine concentration. All test glycine concentrations were 30 s and were followed by a 5 min washout. Oocytes received two consecutive applications of the test glycine concentration to ensure that responses were stable. Alcohols or anesthetics were pre-applied for 1 min alone and then co-applied with glycine for 30 s. All drug applications were immediately followed by a 10 min washout. The test glycine concentration was applied again, and the percent potentiation of the glycine-induced current by the alcohol or anesthetic was calculated for each oocyte. 10 μM $HgCl_2$ was applied for 1 min (in the absence of glycine for the A288C/W407C and A288C/Y410C mutants and in the presence of 100 mM glycine for the A288C/Y406C

and A288C/I409C mutants as previously published in McCracken et al., 2010) and followed by a 15 min washout. Afterward, the maximal glycine concentration was re-applied and the test concentration (EC_{5-10}) was recalculated. The potentiation of glycine-induced current by the alcohol or anesthetic was then measured again as described above . This protocol was used to test the effects, in both the resting and open/desensitized states, of 22 mM butanol and 0.6 mM isoflurane, which corresponds to approximately two times the minimal alveolar concentration (MAC) for these drugs.

Data Analysis. For each receptor tested, repeated-measures t-tests were used to detect differences between pre-$HgCl_2$ and post-$HgCl_2$ conditions (i.e. before and after cross-linking). GraphPad PRISM software (San Diego, CA) was used for all analyses, and statistical significance was determined at $p < 0.05$.

Protein Extraction

Individual oocytes were manually isolated and injected with 1.5 ng/nl cDNA as described above. Injected oocytes were incubated for 5-7 days at 13°C. Groups of 25 oocytes were pooled for each condition. Those included in the cross-linked condition were treated with 0.5 % H_2O_2 by bath perfusion as described in McCracken et al. 2010 and as described for the $HgCl_2$ application above in the electrophysiology experiments. Following a 15 min washout, oocytes were homogenized in 1 mL of Wash Buffer (0.1M EDTA, pH 7.5; 0.1 M EGTA, pH 7.5; 2M NaCl; 0.1M NaH_2PO_4, pH 7.5) with protease inhibitors (5 mM benzamidine; 15 mM iodoacetamide), and then centrifuged at 4°C for 30 min at 13500 rpm. The supernatant was removed, and 250 μL of Extraction Buffer

(wash buffer described above + 2% Triton + 5 mM benzamidine + 15 mM iodoacetamide) was added. The pellet was resuspended, rotated at 4°C for 2 hrs, and then centrifuged at 4°C for 30 min at 13500 rpm. The supernatant was removed and saved as the protein extract. Similar protein extract protocols using *Xenopus* oocytes have been described previously by Bali et al. (2009).

Immunoblotting

Equal amounts of soluble proteins extracted from oocytes were resolved by SDS-PAGE and electrotransferred to polyvinylidene fluoride membrane in a buffer containing 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, and 20% (v/v) methanol for 1 h at 18 V. Membranes were briefly rinsed in a buffer containing 10 mM Tris HCl (pH 8.0), 150 mM NaCl and 0.01% (v/v) Tween-20 (TBS-T), and then incubated at room temperature for 1 h in TBS-T containing 5% (w/v) skimmed milk powder to block non-specific binding of antibodies. Incubation of membranes with specific primary antibody for GlyR α1 (AbCam, Cambridge, England) was performed in TBS-T containing 1% skimmed milk and 1% BSA powders (w/v) for 2 h at room temperature, followed by three 10 min washes with TBS-T alone. Membranes were then incubated for 1 h at room temperature with an appropriate secondary antibody conjugated to horseradish peroxidase (HRP), diluted in TBS-T /1% (w/v) skimmed milk and BSA powders, followed by three 5 min washes with TBS-T. After the final wash, blots were immediately developed by applying the Enhanced Chemi-Luminescence (ECL) reagent (Pierce Chemical Co., Rockford, IL)

for 2 min, and then a Kodak Image Station 2000MM (Eastman Kodak, Rochester, NY) was used to acquire images.

Protein Identification by Mass Spectrometry

Gel bands of interest were excised and washed 3 times then digested in-gel with modified porcine trypsin protease (Promega, Fitchburg, WI). The digested tryptic peptides were desalted using a Zip-tip C18 (Millipore, Billerica, MA). Peptides were eluted from the Zip-tip with 0.5μL of matrix solution (α-cyano-4-hydroxycinnamic acid, 5 mg/mL in 50% acetonitrile, 0.1% trifluoroacetic acid, 25mM ammonium bicarbonate) and spotted on a MALDI plate.

Mass Spectrometry. MALDI-TOF MS and TOF/TOF tandem MS/MS were performed on AB SCIEX TOF/TOF™ 5800 System (AB SCIEX). MALDI-TOF mass spectra were acquired in reflectron positive ion mode, averaging 4000 laser shots per spectrum. TOF/TOF tandem MS fragmentation spectra were acquired for each sample, averaging 4000 laser shots per fragmentation spectrum on each of the 7-10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions).

Database search. Both the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix Science, Boston, MA) to search the Swiss-Prot database. Searches were performed without constraining protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and

with one missed cleavage also allowed in the search parameters. Protein identification in the mass spectra was performed by Applied Biomics (Hayward, CA).

Quantification of Band Ratios

ImageJ64 was used to process and quantify α1 GlyR-labeled band intensity at approximately 100 kDa and 50 kDa, which corresponded to the loci in which monomeric and dimeric GlyR subunits were identified by MS. These band intensities were then calculated and reported as direct ratios (100:50 kDa), and statistically significant differences in band ratio between the uncross-linked and cross-linked conditions were measured for the representative TM3-4 mutant and the wild-type using repeated-measures t-tests, $p < 0.05$.

Molecular Modeling

A homology model of homo-pentameric GlyRa1 was built by threading the primary GlyR sequence onto the X-ray structure of the eukaryotic glutamate-gated chloride channel (GluCl, PDB ID 3RHW). The cytoplasmic TM3-4 loop was trimmed to match the length of the GluCl template in that region. The modeling and subsequent refinement were essentially as described previously (McCracken et al., 2010). In that the GluCl structure is eukaryotic and, as opposed to our previous models based on GLIC (PDB ID 3EAM), is ligand-gated in the ligand-binding domain rather than by protons. In addition, the sequence identity and similarity between GlyR and GluCl is higher that with GLIC (McCracken, et al., 2010). In contrast to the alignment of GlyR with nAChR (PDB ID

2BG9), there are no gaps in the alignment in the TM2-3 loop (Ernst, 2005) and the similarity of residues in TM4 is evident. The refined model was used to mutate residues of interest to cysteine and measure center-to-center distances between sulfur atoms.

RESULTS

Previous studies using cysteine substitution and alkyl thiol reagents have shown that residues in TM4 are water-accessible and suggest that these residues, along with others from TM1-3, including A288, may contribute to a water-filled, intra-subunit drug binding pocket (Jenkins et al., 2001; Lobo et al., 2006; Lobo et al., 2008). In addition, studies using cysteine mutagenesis and cross-linking agents have begun to investigate the proximity of A288 in TM3 to residues in TM4 and provide evidence that Y406, W407, I409, and Y410 in TM4 are able to form cross-links with A288 in TM3, which supports the assignment of these residues as intra-subunit facing (McCracken et al., 2010). Therefore, the purpose of the present study was to determine whether cross-linking A288C in TM3 with intra-subunit facing residues in TM4 alters alcohol or volatile anesthetic enhancement of α1 GlyR function.

The four double cysteine mutants (A288C/Y406C, A288C/W407C, A288C/I409C, and A288C/Y410C) previously shown to form cross-links in McCracken et al. (2010) as well as the corresponding single mutants were each expressed in Xenopus laevis oocytes. Two-electrode voltage clamp electrophysiology was used to measure glycine-induced current, and alcohol and volatile anesthetic effects were measured before and after the application of the cross-linking agent $HgCl_2$, which reacts with accessible pairs of cysteines to form intermolecular S-Hg-S dimers when the residues are in proximity to one another and located on opposing faces of adjacent helices (Struthers et al., 2000; Soskine et al., 2002). The glycine concentration-responses of the single and double cysteine mutants were reported (Lobo et al., 2006; McCracken et al., 2010), and

although the mutations result in some degree of altered glycine sensitivity (Table 3.1), all of the mutants formed functional GlyRs.

First, we investigated whether the cross-linking of A288C in TM3 with intra-subunit facing residues in TM4 alters alcohol enhancement of GlyR function. We measured the potentiation of submaximal (EC_{5-10}) glycine-induced current by 22 mM butanol before and after application of the cross-linking agent $HgCl_2$. As described in McCracken et al. (2010), 10 μM $HgCl_2$ was applied to the A288C/W407C and A288C/Y410C double mutants and corresponding single mutants in the resting state (absence of glycine) (Figure 3.1A). However, agonist-induced rotation of TM3 is necessary for the formation of cross-links in the A288C/Y406C and A288C/I409C, so for these double mutants and the corresponding single mutants, 10 μM $HgCl_2$ was applied in the presence of maximal glycine (Figure 3.1B). We found that while the application of $HgCl_2$ had no significant effect on butanol modulation of submaximal glycine response in the wild-type or single mutants, butanol modulation was significantly decreased in all four double cysteine mutants following the application of $HgCl_2$ (Figure 3.1A and B).

Similarly, we examined whether the cross-linking of A288C in TM3 with intra-subunit facing residues in TM4 alters volatile anesthetic enhancement of GlyR function. We compared the potentiation of submaximal (EC_{5-10}) glycine current by 0.6 mM isoflurane before and after the application of $HgCl_2$. As described above, $HgCl_2$ was applied to the A288C/W407C and A288C/Y410C double mutants and corresponding single mutants in the resting state (absence of glycine), whereas $HgCl_2$ was applied in the presence of maximal glycine for the A288C/Y406C and A288C/I409C double mutants

Receptor	Glycine EC_{50} (mM)
WT	0.3 ± 0.1
A288C	2.8 ± 0.8
Y406C	3.8 ± 0.1
W407C	0.7 ± 0.1
I409C	0.4 ± 0.1
Y410C	0.5 ± 0.1
A288C/Y406C	6.5 ± 1.5
A288C/W407C	6.0 ± 0.2
A288C/I409C	4.4 ± 2.8
A288C/Y410C	26 ± 1.6

Table 3
Glycine EC_{50} values for wild-type and mutant TM3-4 glycine receptors. All values represent mean ± SEM from 4-11 oocytes. Adapted from McCracken et al., 2010.

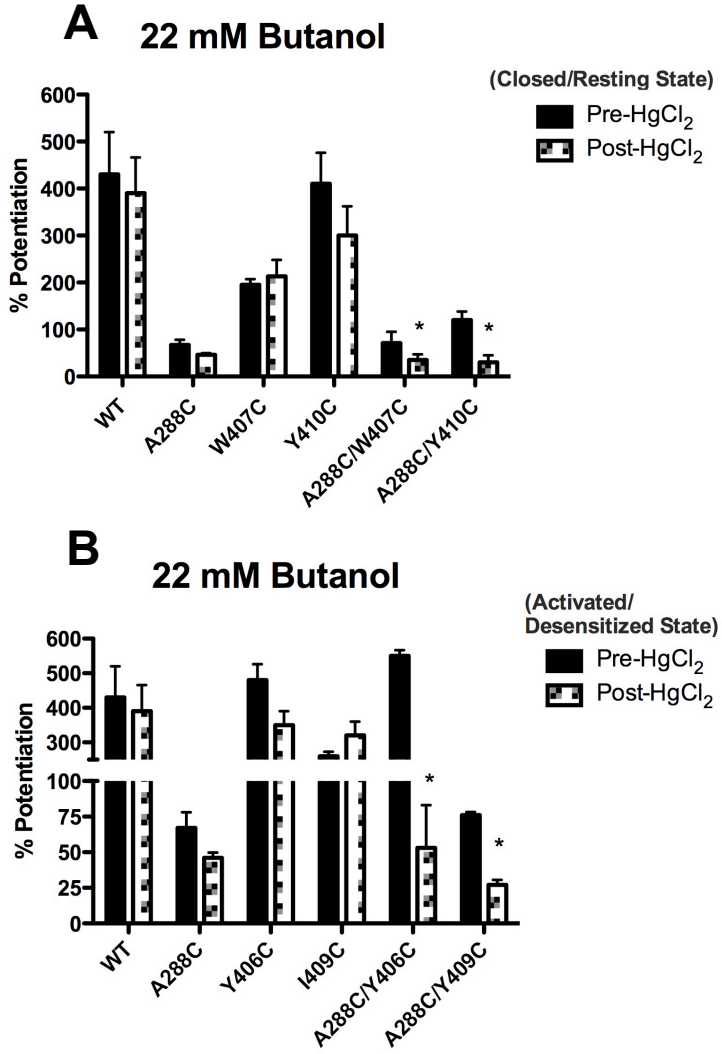

Figure 3.1. Alcohol potentiation of $EC_{5\text{-}10}$ glycine response in TM3-4 mutant GlyRs. A) As described in McCracken et al. (2010), 10 μM $HgCl_2$ was applied to the A288C/W407C and A288C/Y410C double mutants in the absence of glycine (i.e. the resting state) and produced evidence of cross-linking. Accordingly, the butanol effect on these double mutants and the corresponding single mutants was compared before and after 10 μM $HgCl_2$ was applied alone. B) However for the A288C/Y406C and A288C/I409C double mutants, cross-linking was only observed when 10 μM $HgCl_2$ was applied in the presence of 100 mM glycine (i.e. the activated/desensitized state), so the these double mutants and the corresponding single mutants were tested under similar conditions. All values represent mean ± SEM from 4-5 oocytes. Repeated-measures t-tests were used to detect differences between the Pre-$HgCl_2$ and Post-$HgCl_2$ conditions for each receptor. *$p<0.05$

and corresponding single mutants. Following the application of $HgCl_2$, isoflurane potentiation was nearly abolished in all four of the double cysteine mutants, but not in the wild-type or single mutants (Figure 3.2A and B).

Next, we addressed the possibility that the diminished butanol and isoflurane effects in the double mutants following $HgCl_2$ application was due to Hg2+ binding to each of the substituted cysteines and forming S-Hg-Cl bonds, rather forming a cross-link between TM3 and TM4 and reducing the ability of butanol or isoflurane to bind and produce an effect at an intra-subunit cavity within in the TM domain of the GlyR. Although this possibility is unlikely given that the butanol and isoflurane effects were not significantly altered in the single mutants, we measured butanol and isoflurane modulation in the A288C/Y406C and A288C/I409C mutants when $HgCl_2$ was applied in the absence of glycine, which has been previously shown not to produce functional effects or, presumably, cross-links. Accordingly, we found that in the A288C/Y406C mutant, the potentiation of EC_{5-10} glycine response by 22 mM butanol was not significantly altered following the application of $HgCl_2$ in the absence of glycine. In addition, the application of $HgCl_2$ alone did not significantly alter potentiation by 0.6 mM isoflurane. Similarly, $HgCl_2$ alone had no significant effect on butanol or isoflurane modulation of the A288C/I409C double mutant (data not shown). Taken together, our findings suggest that the formation of a cross-link between A288 in TM3 and residues in TM4 reduces the ability of alcohols and volatile anesthetics to bind and produce an effect and further supports the participation of these residues in a common binding cavity. However, it remains uncertain whether these cross-links are formed between residues

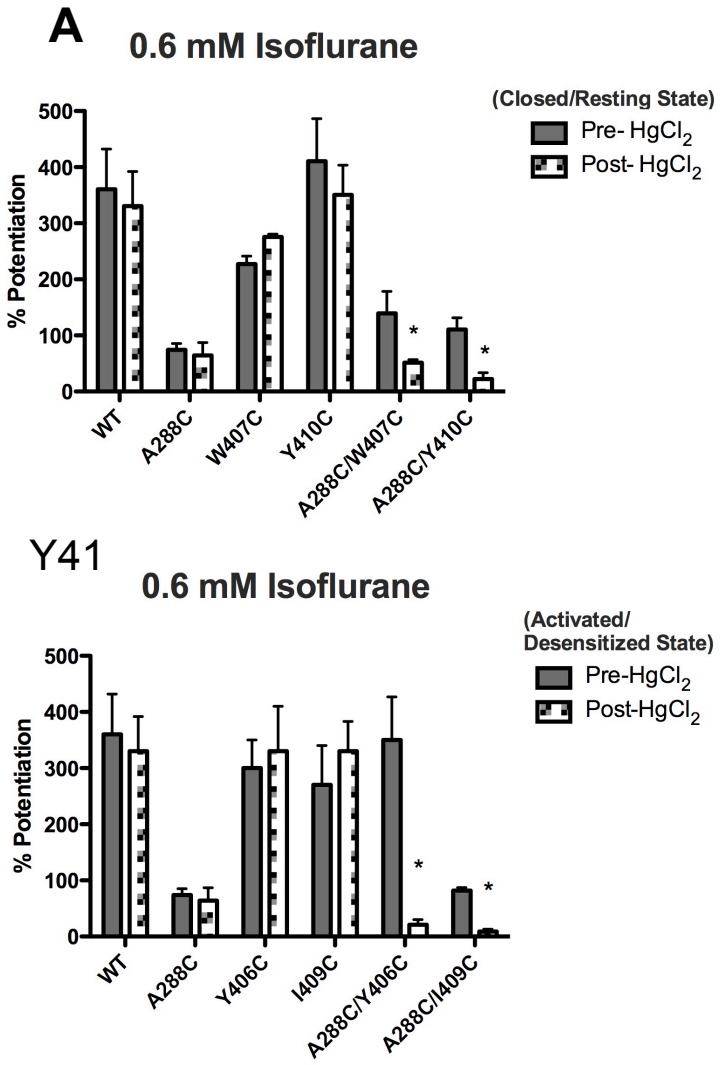

Figure 3.2. Anesthetic potentiation of $EC_{5\text{-}10}$ glycine response in mutant TM3-4 GlyRs. A) In accordance with the butanol experiments, 10 μM $HgCl_2$ was applied to the A288C/W407C and A288C/Y410C double mutants and corresponding single mutants in the resting state (absence of glycine), and the potentiation of submaximal glycine responses by 0.6 mM isoflurane was compared before and afterward. B) For the A288C/Y406C and A288C/I409C double mutants and corresponding single mutants, $HgCl_2$ was applied in the activated/desensitized state (presence of 100 mM glycine). All values represent mean ± SEM from 4-5 oocytes. Repeated-measures t-tests were used to detect differences between the Pre-$HgCl_2$ and Post-$HgCl_2$ conditions for each receptor. *$p<0.05$

within the same subunit (intra-subunit) or between subunits (inter-subunit), and ultimately whether these residues are participating in common alcohol-binding cavity within or between GlyR subunits.

We used the homology model of GlyR with the residues of interest substituted with cysteine to determine potential sites for cross-linking (Figure 3.3). Distances between S-S with cysteines substituted at I229 (TM1), S267 (TM2), A288 (TM3), and Y406 (TM4) in two adjacent subunits ranged from about 7 to 12 Angstroms. An ideal S-S disulfide bond is approximately 2 Angstroms. The use of $HgCl_2$ to form an S-Hg-S bond extends that final bond distance to approximately 5 Angstroms. Obviously, some rotation or bending of the helical segments would be required to accommodate the experimental cross-linking results.

To further investigate these possibilities, we extracted GlyR protein from oocytes injected with wild-type GlyRs or a representative double cysteine TM3-4 mutant from above (A288C/Y410C), which has shown evidence of cross-link formation using either $HgCl_2$ or H_2O_2 (McCracken et al., 2010). We then used immunoblotting with a GlyR-specific antibody (Figure 3.3) and verified protein identification by mass spectrometry (MALDI-TOF/TOF) (Table 3.2). The differences in 100:50 kDa band ratios were quantified and compared between uncross-linked and cross-linked conditions for the representative TM3-4 mutant and the wild-type. Wild-type GlyR subunits typically measure about 50 kDa. Therefore, we hypothesized that if cross-linking occurred between two adjacent subunits, then we would observe an increase in the presence of

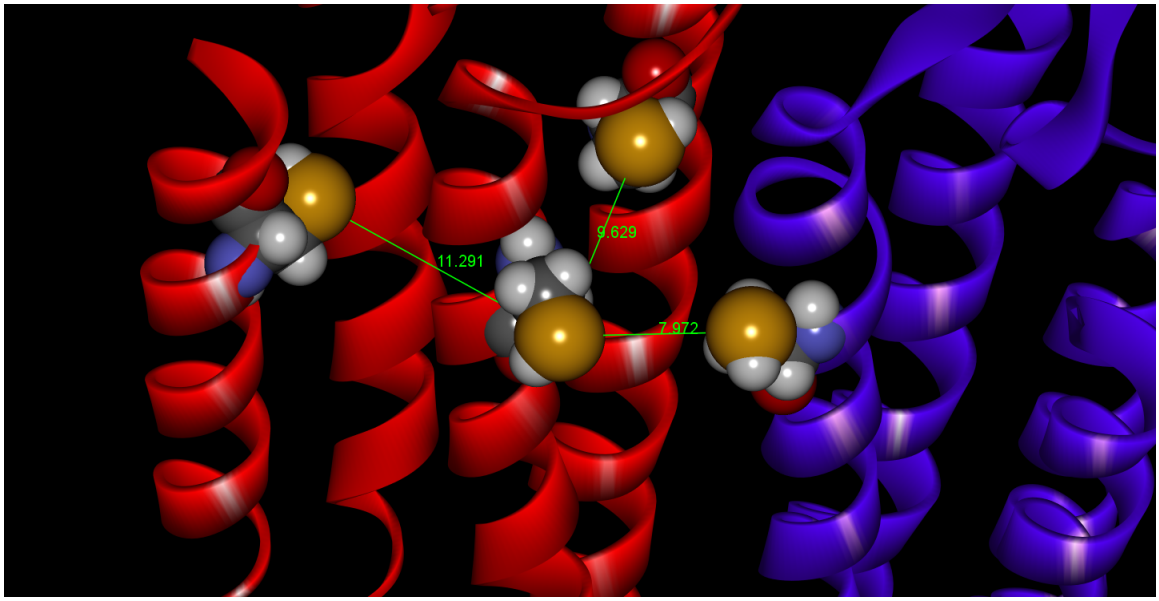

Figure 3.3. Homology Model of 3D structure of portions of GlyR TM1-4. Based on the invertebrate Caenorhabditis elegans glutamate-gated chloride channel (GluCl), as described in Methods, distances are between S-S with cysteines substituted at I229 (TM1), S267 (TM2), A288 (TM3), and Y406 (TM4) in two adjacent subunits (red and blue). An ideal S-S disulfide bond is theoretically about 2 Å, however the use of $HgCl_2$ to form an S-Hg-S bond extends that final bond distance to approximately 5 Å. The distances between S-S with cysteines substituted at the respective positions noted above ranged from about 7-12 Å.

GlyR-labeled 100 kDa band, and an increase in the ratio of 100:50 kDa bands. Conversely, we expected no change for cross-linking within a subunit. We did not find any significant differences in the 100:50 kDa band ratios between the uncross-linked or cross-linked conditions for either the TM3-4 mutant or the wild-type (3.4). This is consistent with previous homology modeling (Bertaccini et al., 2005; Unwin et al., 2005) and biochemical cross-linking studies suggesting that the TM4 residues of interest: Y406, W407, I409, and Y410 form intra-subunit cross-links with A288 of TM3 from the same subunit (McCracken et al., 2010) and provides evidence that these residues may participate in a common drug-binding cavity within a GlyR subunit.

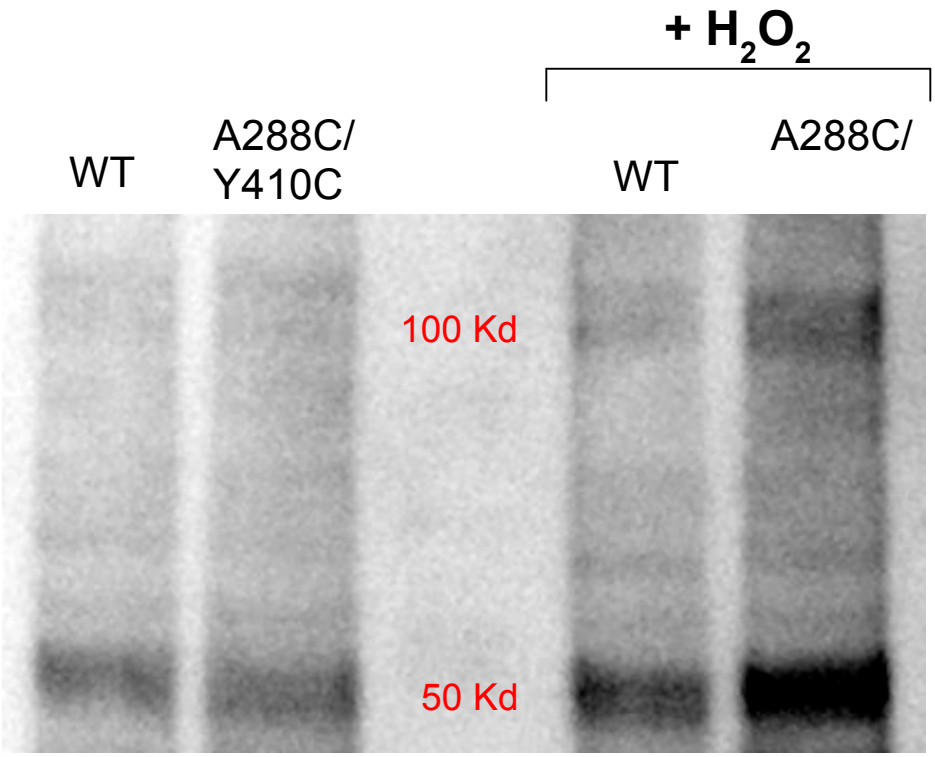

Figure 3.4. Immunoblot for WT and mutant TM3-4 GlyRs. 100 kDa and 50 kDa α1 GlyR-labeled bands in the wild-type and A288C/Y410C mutant in the uncross-linked vs cross-linked (+H_2O_2) conditions. 0.5% H_2O_2 was applied by bath perfusion as previously described (McCracken et al., 2010).

	Molecular Weight (kDa)	Protein Score C.I. %
GLRA1	100-105	57
GLRA1	100	60
GLRA1	50-55	89
GLRA1	48-50	78

Table 4
Protein Identification of Mutant TM-3-4 Glycine Receptor. All values are from a representative uncross-linked A288C/Y410C mutant sample determined by MALDI-TOF/TOF.

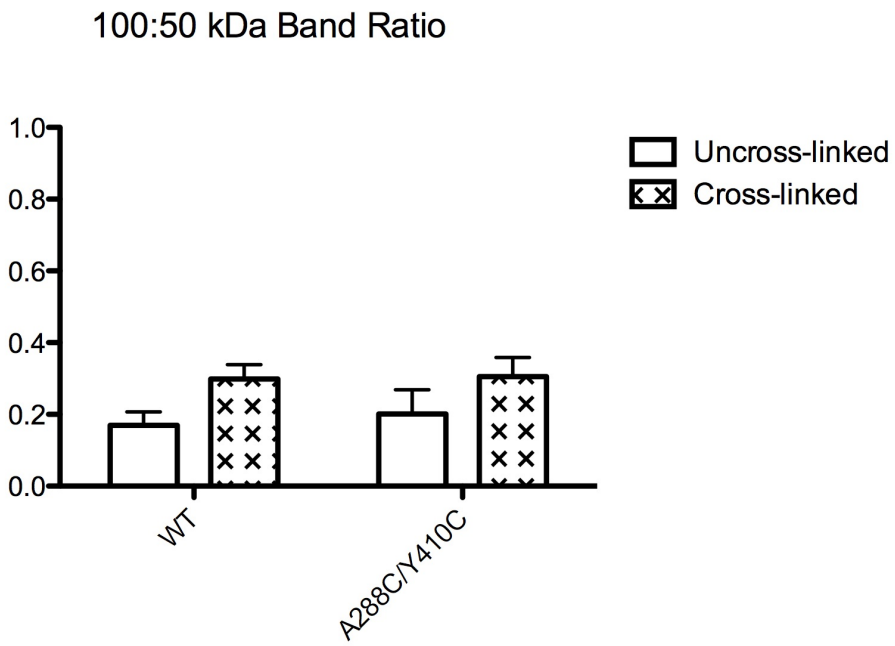

Figure 3.5. Quantification of Band Ratios for WT and mutant TM3-4 GlyRs. The100:50 kDa band ratio did not increase for the A288C/Y410C double mutant or the wild-type when cross-linked. Immunoblot images were processed using ImageJ64 software, and the band intensity was taken and reported as a direct ratio. 0.5% H_2O_2 was applied by bath perfusion as previously described (McCracken et al., 2010). All values represent mean ± SEM. Repeated-measures t-tests were used to detect differences between the uncross-linked vs cross-linked (+H_2O_2) conditions for each receptor, $p>0.05$.

DISCUSSION

Our findings suggest that the formation of a cross-link between residue 288 in TM3 and residues Y406, W407, I409, and Y410 in TM4 reduces the ability of alcohol and volatile anesthetics to bind and produce their effects at an intra-subunit cavity in the GlyR. Although the crystal structure of the glycine receptor has not been solved, our results are consistent with the recent x-ray structure of desflurane bound at an intra-subunit cavity in the bacterial homologue GLIC (Nury et al., 2011). More recently, photolabeling studies demonstrated the competitive binding of Propofol and AziPm at residues corresponding to the intra-subunit pocket identified in the GLIC crystal structure (Jayakar et al. 2013; Chiara et al., 2014), which further supports an intra-subunit site of action for anesthetics. However, the interaction of anesthetics and alcohols with GLIC, and the other ligand-gated ion channels, may involve more than a single pocket bounded by the four TM regions. The high-resolution structure of GLIC with desflurane and propofol shows a "linking tunnel," which connects inter- and intra-subunit cavities (Nury et al., 2011).

The position of A288C in (Figure 3.3) is facing more into the inter-subunit space than toward the intra-subunit cavity. The model shows that the distances between A288C in TM3 of one subunit and residues in TM4 of the adjacent subunit are so great that the receptor would be considerably distorted by the cross-linking, and presumably, rendered non-functional. A more likely possibility is that the two helical turns at the extracellular end of TM3 are flexible. A somewhat extreme example is a “spring model” used by Otero-Cruz et al. (2007) to explain the effect of substitutions in TM3 of in the related

nAChR. We previously considered the effect of a 100 degree rotation of TM3 during the opening transition to explain experimental cross-linking of TM3 with residues in TM4 (McCracken et al., 2010). Currently, there are many other examples of experimental evidence for specific motion of TM3 or a more global motion within the subunit that changes the accessibility of reagents to the intra-subunit cavity (Jung et al., 2005; Bali et al., 2009; Yoluk et al., 2013).

Another uncertainty is the position of TM4 in these channels (Bertaccini et al., 2010). Studies using voltage-clamp fluorometry have reported differences between the GlyR α_1 and α_3 subunits with respect to the relative orientation and proximity of TM3 and TM4, whereby fluorescence-tagged TM3 and TM4 residues in the α_1 subunit exhibited intra-subunit facing orientation under glycine-dependent conditions whereas homologous α_3 residues displayed evidence of differing orientation (Han et al., 2013). High resolution structures of GLIC, ELIC and GluCl find TM4 to be tilted away from the other three TM regions (Bocquet et al., 2009; Hilf and Dutzler, 2009; Hibbs and Gouaux, 2011) and the intra-subunit binding site for desflurane in GLIC appears to be formed primarily by TM1-3 (Nury et al., 2011). It is possible that the tilt of TM4 away from the other TMs allows it to be partially or completely surrounded by lipid (Baenziger and Corringer, 2011). However, data from cross-linking experiments in the GlyR show that TM3 and TM4 are in sufficient proximity to allow cross-linking not only with $HgCl_2$, but in the case of A288C/Y410C, also with an oxidizing agent (McCracken et al., 2010). Propyl methanethiosulfonate labeling experiments indicate that W407C, I409C, Y410C

and K411C are accessible to this reagent and are likely exposed to water, which is consistent with this region of TM4 contributing to a water-filled cavity (Lobo et al., 2006). It seems likely that TM4 has considerable flexibility and may approach the other TMs, perhaps during gating, which is supported by the observation that cross-linking for A288C/Y406C and A288C/I409C are only observed if the receptor is activated by glycine (McCracken et al., 2010). Moreover, it is tempting to speculate that alcohols and anesthetics are able to stabilize the open state of the GlyR given this state creates a closer orientation of TM4 to the other TMs, which would allow for stronger binding to an intra-subunit pocket formed by all four TM segments.

In summary, we found that the formation of a cross-linking between A288 in TM3 with residues Y406, W407, I409, or Y410 in TM4 reduces the ability of alcohol or anesthetic to enhance GlyR function. Moreover, using immunoblotting we provide evidence that this cross-linking appears to occur within a subunit, suggesting that TM3 and TM4 likely participate in an intra-subunit alcohol/anesthetic binding cavity in the GlyR.

CHAPTER IV: DISCUSSION AND IMPLICATIONS

In the absence of a GlyR crystal structure, the orientation of the TM amino acids critical for alcohol and anesthetic action have been controversial, and ultimately the positioning of the putative drug binding cavity within the GlyR has remained uncertain. The purpose of this project was to investigate whether biochemical cross-links are formed between residues within the same subunit (intra-subunit) or between subunits (inter-subunit), and ultimately to determine whether these residues are participating in a common alcohol/anesthetic binding cavity within or between GlyR subunits (or both).

We found that the formation of a cross-link between I229 in TM1 and A288 in TM3 reduced the ability of butanol or isoflurane to modulate GlyR function. Similarly, we have also shown that residues Y406, W407, I409, and Y410 in TM4 are able to form cross-links with A288 in TM3 (McCracken et al., 2010), and this cross-linking between TM3 and residues in TM4 in the present study also reduced the ability of alcohol or anesthetic to bind and produce its effect. Moreover, we found specific evidence that cross-linking between TM1 and TM3 appears to be inter-subunit, but intra-subunit for TM3 and TM4, suggesting that TM1-3 may participate in an alcohol and anesthetic binding cavity between GlyR subunits while TM3-4 may contribute to a binding cavity within a subunit. Taken together, our findings are in general agreement with the recent "multisite" hypothesis positing that alcohol and anesthetics act at both inter- and intra-subunit sites on ligand-gated ion channels. This hypothesis has been generated largely by mutagenesis studies in the GLIC, ELIC, and GluCl homologues, molecular simulations,

and newer homology modeling based on the known prokaryotic/invertebrate structures. It expands upon the initial structures co-crystallized with drug molecules bound at either an intra-subunit or inter-subunit cavity (Howard et al. 2011; Murail et al., 2012; Spurny et al., 2013; Yoluk et al., 2013).

Although the general structural features of pentameric ligand-gated ion channels, including the GlyR and other members of the Cys-loop family, have been established and fairly well accepted, especially since the emergence of the 4Å resolution structure of the *Torpedo* nAChR, technical challenges have created barriers in membrane protein crystallography limiting the advancement and production of x-ray structures for the mammalian ligand ion channels (Carpenter et al., 2008), particularly GlyRs and $GABA_A$Rs. Despite these challenges, the use of homology modeling combined with studies using mutagenesis and alkyl thiol reagents and/or oxidizing agents have been reasonably successful over the past 10+ years at helping to identify critical amino acid residues for alcohol and anesthetic action at multiple sites throughout the GlyR and its relatives. Primarily, residues in the TM segments have received the most attention and support (Mihic et al., 1997; Mascia et al., 2000; Lobo et al., 2006; Harris et al., 2008; Li et al., 2010; Duret et al., 2011; Borghese et al., 2012), but also sites such as A52 in the N-terminal domain (Davies et al., 2004; Crawford et al., 2007) and K385 the intracellular loop linking TM3 and TM4 have been proposed to play a role in alcohol modulation (Yvenes et al., 2003; Yvenes et al., 2008).

Recently, the known crystal structures for the two prokaryotic homologues, ELIC GLIC (Hilf and Dutzler, 2008; Hilf and Dutlzer, 2009; Bocquet et al., 2009), and the

invertebrate GluCl (Hibbs and Gouaux, 2011) have provided improved templates and better informed homology modeling for the study of GlyRs and related mammalian ion channels. These homologues and their known structures have also advanced the study of structure-function in the alcohol and anesthetic fields. For example, the determination of atomic-resolution anesthetic-bound and alcohol-bound GLIC structures (Nury et al., 2011; Sauguet et al., 2013) as well as an anesthetic-bound ELIC structure (Spurny et al., 2013) have recently begun to help further our understanding of the sites of alcohol and anesthetic action in the related ion channels and improve the characterization of drug binding. And while these known structures have provided solutions to previous alignment quandaries and have helped to pinpoint relevant sites for putative binding cavities with greater efficiency, these known prokaryotic sequences and their x-ray structures, in reality, also still represent models of the GlyR and related proteins found in higher order organisms, despite reasonably high predictive validity. As such there are some differences worth noting between the mammalian anion-selective channels such as the GlyR and $GABA_AR$ and the prokaryotic channels GLIC and ELIC.

First, GLIC is a cation-sensitive channel gated by protons, which contains a short, truncated-like intracellular loop domain that differs from its mammalian homologues, (Bocquet et al., 2009) and possesses some unique pharmacological features. Both general anesthetics and ivermectin inhibit GLIC function (Weng et al., 2010; Duret et al., 2011), which is opposite to the potentiating effects of these drugs on wild-type GlyRs and $GABA_A$. In addition, wild-type GLIC is insensitive to pharmacologically relevant concentrations of ethanol, however Howard et al. (2011) demonstrated potentiation with

higher, anesthetic-range concentrations of ethanol or methanol while longer chain *n*-alcohols produced inhibition. Notably though, more pharmacologically relevant potentiating drug effects have been a reported from a GLIC chimera harboring the human α_1 GlyR TM domain. Like wild-type GlyRs, a variety of modulators potentiate the function of this chimera, including alcohols, anesthetics, and ivermectin (Duret et al. 2011), underscoring the obligatory role of the GlyR TM domain in the potentiating effects of these allosteric modulators. Similarly, ELIC is also a cation-selective ion channel, whose atomic-resolution structure was determined prior to that of GLIC (Hilf and Dutzler, 2008), but difficulty identifying agonists to gate the channel made GLIC more popular and practical for physiological studies. High μM-mM concentrations of GABA, along with other primary amines, have since been shown to behave as agonists at ELIC (Zimmermann and Dutzler, 2011), while ethanol and anesthetics inhibit ELIC function (Spurny et al., 2013). Given these characteristics, GLIC and ELIC may be the closest model for the human nAChR, rather than GlyR or $GABA_A$Rs.

Nevertheless, recent co-crystallization studies in GLIC and ELIC with anesthetics and alcohols (or their derivatives) have provided valuable structural insight to help better understand how and where these drugs are interacting with related human ligand-gated ion channels, including the GlyR. In the initial finding by Nury et al. (2011), both the volatile anesthetic desflurane and the intravenous anesthetic propofol were reported to occupy an intra-subunit cavity. Subsequently, an ethanol-sensitized GLIC variant was also co-crystallized with ethanol, bromoethanol, and bromoform, which revealed primary occupancy of an inter-subunit binding cavity, although in the ethanol-sensitized variant,

both bromo-ethanol and bromoform were retained in the intra-subunit cavity as well (Sauguet et al., 2013). Moreover, the crystallization of ELIC in complex with bromoform resulted in the observation of anomalous density in multiple regions of the protein indicative of binding. These predominantly included a hydrophobic pocket in the extracellular domain between β_7-β_{10} strands, the channel pore, and a novel, more deeply situated inter-subunit TM site (Spurny et al., 2013). However, there was no evidence of bromoform retained in the intra-subunit cavity in the case of ELIC, which could reflect the conformational/state-dependent differences between ELIC and GLIC (Spurny et al., 2013). Figure 4.1 provides a general overview of the different general anesthetic binding sites revealed by crystal structures of GLIC and/or ELIC (note that ethanol is unfortunately not included, however it shares many overlapping inter-subunit and intra-subunit sites as discussed above).

Although the present study did not address possible alcohol and anesthetic sites outside of the TM domain, our findings appear to be consistent with a multisite model in the GlyR. In functional studies of GLIC, the delineation of a potentiating inter-subunit cavity and an inhibitory intra-subunit cavity have been proposed (Murail et al., 2012; Sauguet et al., 2013). This was developed largely from the observation that introduction of the single F14'A point mutation in GLIC results not in only potentiation rather than inhibition by alcohol and anesthetics, but at much more relevant concentrations (Howard et al., 2011; Sauguet et al., 2013). Moreover, molecular simulations have expanded upon this and even generated models for GlyRs and $GABA_A$Rs (Murail et al., 2012), however inconsistencies among experimental data involving the mutagenesis of conserved sites

directly identified in the anesthetic-bound crystal structures have made the identification and proposal for the intra-subunit inhibitory site in GLIC a bit weaker (Ghosh et al., 2013). Furthermore, in $GABA_AR$ experiments similar those conducted in the present study, Borghese et al. (2014) reported evidence of both inter-subunit and intra-subunit alcohol/anesthetic binding, but found no relationship between where the anesthetic binds and the type of modulation they produce (potentiation vs. inhibition). Similarly, the modulation we observed in GlyRs was consistently potentiating in nature for both alcohol and volatile anesthetics. However, future studies may be necessary to further investigate whether a precise relationship exists between where alcohols/anesthetics bind (inter-subunit vs. intra-subunit) in the GlyR and the type of modulation the produce (potentiation vs. inhibition).

In conclusion, we provide evidence that alcohols and anesthetics act at both inter- and intra-subunit cavities in the GlyR, which is generally consistent with multisite models derived from the known sequences and structures of bacterial and other invertebrate homologues. The use of these structures as templates for related higher order ion channels such as the GlyR allows for greater efficiency in the study of alcohol and anesthetic sites and mechanisms. Moreover, their continued use and refinement may one day offer a more viable approach toward the development of novel, safer general anesthetics and more effective treatment options for alcohol abuse and alcoholism.

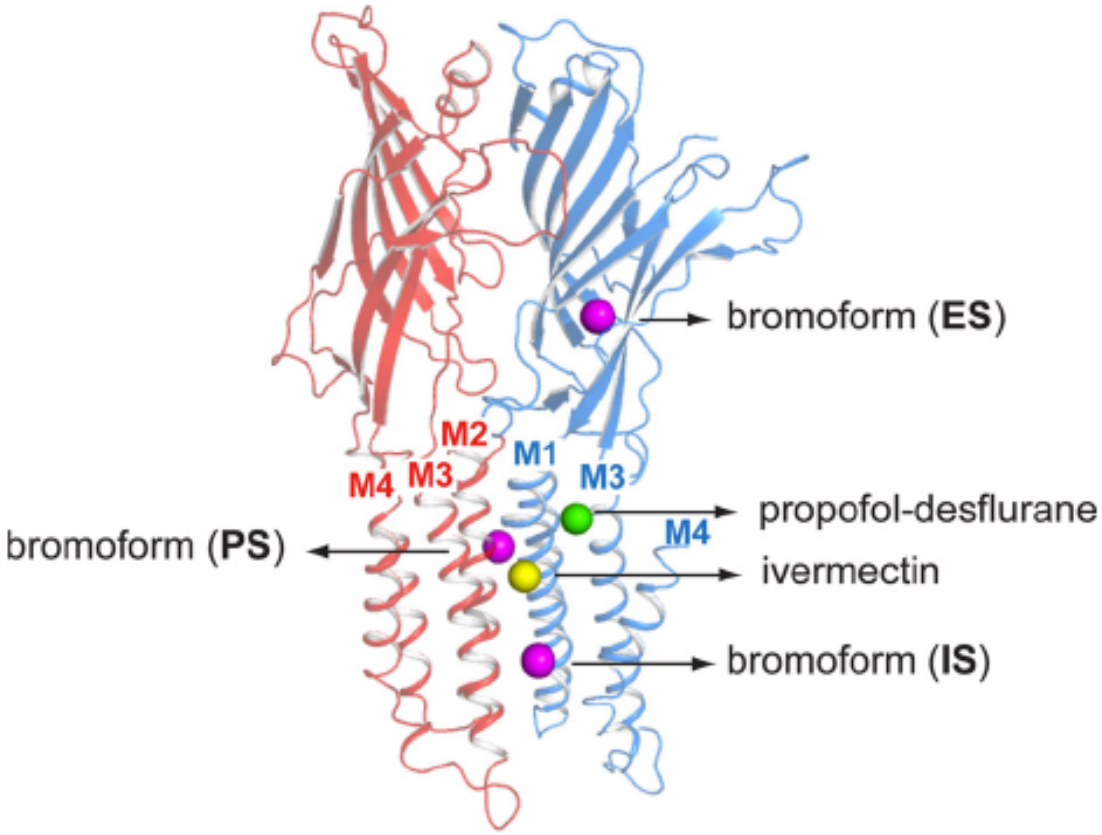

Figure 4.1. Overview of anesthetic binding sites in pentameric ligand-gated ion channels. Graphic representation is based on the known ELIC structure. Two adjacent subunits are shown in red and blue, and drugs placed schematically and labeled accordingly. (ES) = Extracellular site; (PS) = Pore site; (IS) = Inter-subunit site; Note the propofol-desflurane Intra-subunit site is drawn with a representative drug molecule bound, but not labeled. (Adapted from Spurny et al., 2013).

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