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Critical Elements Contributing to the Control of Glycine Receptor Activation and Allosteric Modulation

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Critical Elements Contributing to the Control of Glycine Receptor Activation and Allosteric Modulation

by

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Dedication

I dedicate this dissertation to my mother and my father for helping me reach my goals, teaching me to have an open mind and allowing me to explore and be free, to my son Davor who is the light of my life, and Brady, my partner, my love, who always motivates me to be the best that I can be.

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No one can quite prepare you for the experience of graduate school, but it sure helps to be lucky enough to be supported in this challenge by an amazing mentor and friend. This is what Dr. John Mihic was to me during the years that I spent in his lab. He was often the savior of my sanity and an outstanding source of great ideas and interesting stories. He has helped me stay on track even in the darkest moments, when I thought I was lost and finish line was anything but in sight. I am afraid that no words can do justice to how grateful I am to John for his constant optimism and understanding (I know he surely kept a stash of ibuprofen in his office to alleviate headaches that my data often caused him). I can only hope that I will have the wisdom and aptitude to change the lives of others like he has changed mine.

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working with him in the future and finishing the projects that we have started. My thanks also go to Marsha Berkman who has always brought laughter and life into the lab. She sure was a wonderful person to have around. In addition I thank other helpful individuals from the Waggoner Center for Alcohol and Addiction Research, in particular Dr. Adron Harris and Debbie James.

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Finally I would like to express my appreciation to my family and friends. My son Davor has given me motivation and reason when I couldn't find it anywhere else. Dado and Brady shared me with this immense responsibility for a very long time. For the understanding that they showed, especially towards the end, I will always be grateful. To Brady, my love, I owe so much. He kept me going and believed in me when I had no strength to do so myself. He was a tremendous support and I couldn't have done this without his help and love. My family here and in Serbia has also been a constant reminder of love and support. To my mom and dad, Mirjana and Milorad Todorovic, my brother Ivan, Brady's family, Sue Ellen and Phil Miner, Nick and Mallory – I thank you all for being there and for celebrating my successes with me. I will forever be indebted by the kindness and love that you have all shown me.

Critical Elements Contributing to the Control of Glycine Receptor Activation and Allosteric Modulation

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Jelena Todorovic, Ph.D.

The University of Texas at Austin, 2010

Supervisor: S. John Mihic

Co-Supervisor: John H. Richburg

Glycine receptors (GlyRs) are ligand-gated ion channels (LGICs) that, along with other members of the cys-loop superfamily of receptors, mediate a considerable portion of fast neurotransmission in the central nervous system (CNS). GlyRs are pentameric channels, organized quasi-symmetrically around an ion-conducting pore. Opening of the integral ion pore depends on ligand binding and transduction of this binding signal to the channel gate.

Research presented in this dissertation describes a number of critical electrostatic interactions that play a role in conserving the closed-state stability of the receptor in the absence of ligand, ensuring that receptor activation occurs only upon neurotransmitter binding. These amino acids, aspartic acid at position 97 (D97), lysine 116 (K116),

arginine 119 (R119) and arginine R131 (R131) are charged residues that interact with one another through electrostatic attraction. When D97 is replaced with any other amino acid this destabilizes the closed state of the channel and causes spontaneous GlyR channel opening. I show that restoration of this electrostatic interaction in GlyR bearing double mutations in which the charges are swapped (D97R/R119E and D97R/R131D) markedly decreases this spontaneous current. In addition, I investigate how these residues that interact at the interfaces between receptor subunits affect the efficacies of GlyR partial agonists. My work shows that the partial agonist taurine is converted into a full agonist at both D97R and R131D receptors.

Furthermore, I analyzed the structure of the more extracellular part of the transmembrane (TM) 2 segment that lines the ion channel pore, showing that it is unlikely that this fragment (stretching from T13' to S18') is constrained in a true alpha helical conformation. From this work, using disulfide trapping and whole cell electrophysiology, I conclude that a significant level of flexibility characterizes this part of the TM2 domain. This segment includes residue S267, previously shown to be significant for alcohol and anesthetic actions, as well as residue Q266 that, when mutated, produces a hyperekplexia-like phenotype. The range of movement of residues in this region may therefore play an important role not only in channel gating but also in how modulators of GlyR function exert their actions.

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List of Abbreviations

5-HT_3R……..……..serotonin receptor

a……...……area, amplitude

ANOVA……...analysis of variance

Br^-……...……...bromide ion

Cl^-……...……chloride ion

CNS……..……. central nervous system

cryo-EM……...……..cryo-electron microscopy

DTT……..…….……1,4 dithiothreitol

EC…….……..effective concentration

ECD……...……extracellular domain

ELIC……*Escherichia coli* ligand-gated ion channel

E_m……membrane potential

EtOH...……...……..…….ethanol

F^-……..……..……fluoride anion

$GABA_AR$……...γ-aminobutyric acid receptor (type A)

$GABA_{A\text{-}\rho}R$……...…γ-aminobuturic acid receptor (subtype rho)

GLIC…….....*Gleobacter violaceus* ligand-gated ion channel

GlyR……...glycine receptor

GlyT2……..……...……glycine transporter type 2

H_2O_2…….….hydrogen peroxide

$HgCl_2$……...……..mercury (II) chloride

I^-……Iodine anion

K^+……..……potassium cation

LBD……...…..ligand binding domain

LGIC…….……...ligand gated ion channel

MBS…….……..modified Barth's solution

MTS……..…methane thiosulfonate

nAChR…….....nicotinic acetycholine receptor

nAChBP…….……....nicotinic acetylcholine binding receptor

NMR……..……nuclear magnetic resonance

NO^{3-}……..……...nitrate anion

P_{open}……...……..……channel open probability

PMTS…….…….…….propyl methanethiosulfonate

PTX……..……picrotoxin

REFER…….....rate-equilibrium free energy relationship

SCN^-……...……thiocyanate

TFE……...…….……trifluoroethanol

TM…….……..transmembrane segment

VTA……...…….ventral tegmental area

WT…….……wild type

Zn^{2+}…….……zinc

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GLYCINE RECEPTOR LIGANDS

Agonists:	**Antagonists:**
glycine	strychnine
taurine	nipecotic acid
β-alanine	picrotoxin (picrotoxinin + picrotin)

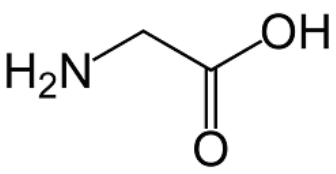
Modulators:

Alcohols (ethanol, propanol, octanol etc.)

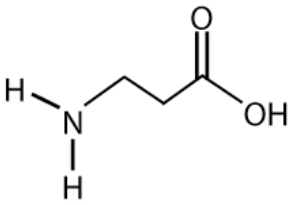
Anesthetics (isoflurane, chloroform, propofol etc.)

Zinc (Zn^{2+})

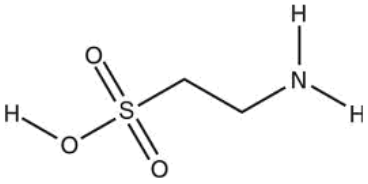
STRUCTURES OF SOME OF THE GLYCINE RECEPTOR LIGANDS



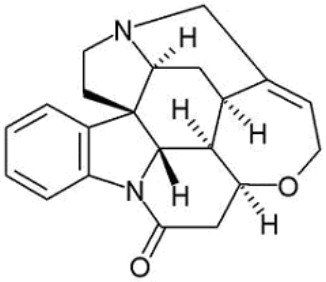
Glycine



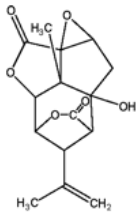
β Alanine



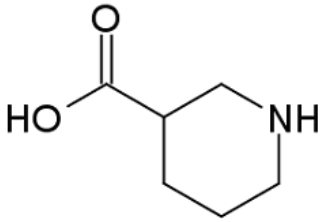
Taurine



Strychnine

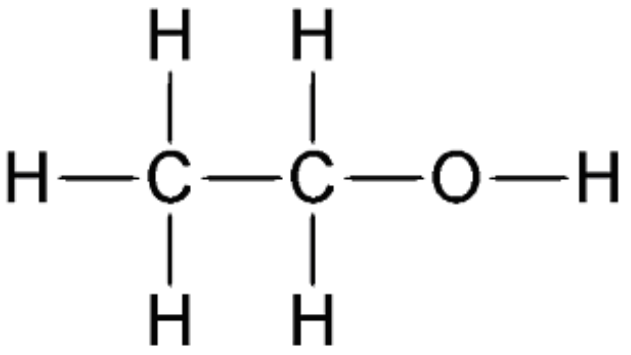


Picrotoxinin (PTX)

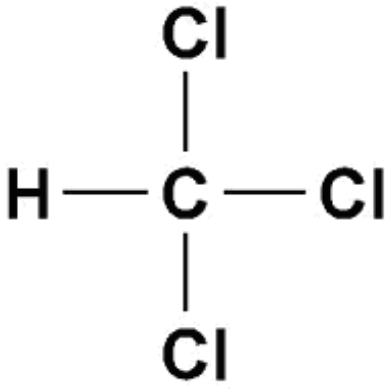


Nipecotic acid

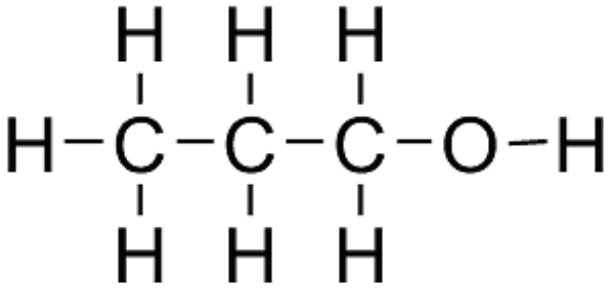
OTHER RELEVANT COMPOUNDS



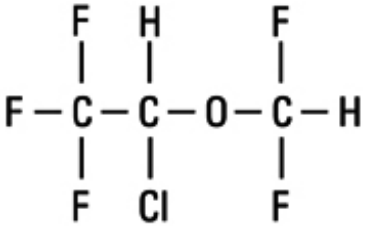
Ethanol



Chloroform



Propanol



Isoflurane

CHAPTER 1: INTRODUCTION

1.1 The glycinergic synapse: background and significance

The central nervous system (CNS) is responsible for thought processes, memory, motor coordination and other functions vital for sustaining body homeostasis. Consisting of the brain and spinal cord it represents an exceptionally complex network of cells that governs human consciousness and understanding of self, as well as other more autonomic functions such as breathing, digestion and heart rate control. With trillions of cells and even more neuronal connections these neuronal networks are challenging to explore and decipher. The complexity of specialized contacts of neuronal "chatter", synapses, are governed by the abundance of neurotransmitters that mediate chemical communication among neurons. These formations are believed to hide the key to the understanding of numerous CNS disorders (**Figure 1.1:glycinergic synapse**). Anesthetics, alcohols and various drugs of abuse are just some of the compounds that are known to exert their effects by modulating the function of neuronal proteins.

The glycine receptor (GlyR) plays a significant role in fast neuroinhibition in the CNS. Although numerous studies have localized this integral membrane protein in higher brain regions (egs, hypothalamus, nucleus accumbens and cerebellum), it is thought to play its major role by acting on the neurons of the spinal cord, brain stem and retina (Lynch, 2004). Numerous studies have shown that many pharmacologically important agents and substances of abuse modulate the function of GlyRs, in this way affecting the balance between the inhibitory and excitatory influences in the CNS. The

major role of GlyRs in postsynaptic inhibition makes them an attractive target for drug development.

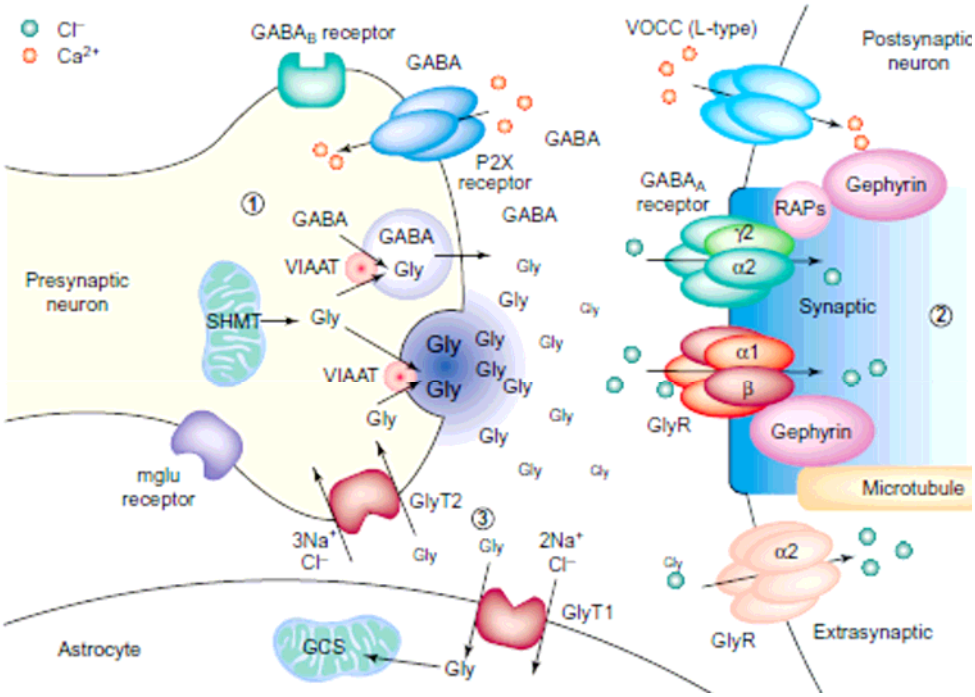


Figure 1.1 The glycinergic synapse. Some of the molecular constituents playing important roles in the inhibitory synapse are presented here: glycine is released from presynaptic vesicles onto a postsynaptic cell where it activates GlyRs. Gephyrin is a protein organizing heteromeric GlyRs at the membrane surface by acting as a bridge between the receptor's β subunit and cytoskeleton. GlyT1 and GlyT2 are transporters responsible for reuptake of glycine from the synapse. *(Adapted from Laube et al., TRENDS in Pharmacological Sciences, Vol 23 No.11)*

1.2 Molecular biology of glycine receptor

The GlyR belongs to the cys-loop superfamily of receptor subunits, which include the excitatory nicotinic acetylcholine receptor (nAChR) and serotonin-3 receptor (5-HT_3R), as well as other inhibitory receptors, γ aminobutyric acid type A ($GABA_A$) and γ aminobutyric acid type A, subclass rho ($GABA_{A-\rho}$). These pentameric proteins are characterized by their dependence on ligand binding for the opening of their integral ion channels and are recognized by an invariant cys-loop structure in the extracellular domain (ECD). Each of the five GlyR subunits consists of a large ECD, a transmembrane domain consisting of 4 segments (TM1-4), a long intracellular loop connecting the TM3 and TM4 segments and a short C-terminal domain located on the extracellular side (Lynch, 2004). Agonist binding pockets are located within the ECD. When 5 subunits co-assemble to form a receptor, each subunit contributes its TM2 segment to form the pore, which also constitutes the ion selectivity barrier of the channel. Thus far 5 subunit encoding GlyR genes have been identified: α1-4 and one β subunit (Betz et al., 1991).

The amino acid glycine is an endogenous ligand at GlyR. It controls fast neurotransmission in the central nervous system by activating its channel at glycinergic synapses and allowing Cl^- ions to pass across the postsynaptic membrane. If Cl^- influx occurs as a result of GlyR opening (when the cell membrane potential is less negative than E_{Cl}-) this will hyperpolarize the membrane and inhibit action potential generation. In the special case of "shunting inhibition" glycine balances excitatory and inhibitory tones, but not by simple subtractive hyperpolarization. In this case GlyR activation will result in the reduction of depolarization caused by the excitatory input without causing large

outward Cl^- current (as is the case when the Cl^- gradient is small or non-existent; $E_{Cl^-} \approx E_m$) (Price et al., 2009). Prenatally glycine can play a different role and depolarize the postsynaptic membrane. This is due to the difference in Cl^- concentrations during embryogenesis. Higher intracellular concentrations of chloride during this stage will cause efflux of this anion in order to reach E_{Cl^-} (due to the change in Cl^- gradient). As neurons mature they express a specialized transporter (K^+-Cl^- co-transporter) that will drive down intracellular Cl^- concentrations (Stein and Nicoll, 2003), resulting in the transition of both $GABA_A$ and glycine receptors to the inhibitory roles they play in adults.

Local movements of different segments in the loops of the ligand binding domain (LBD) are believed to initiate the transduction of the glycine binding signal to residues located in TM2 that constitute the channel gate. Although a recent crystal structure and refined model of the nAChR (Unwin, 2005) resulted in a broader understanding of the structure of the cys-loop ligand-gated ion channels much remains to be determined to gain a full understanding of the steps involved in conformational movements involved in signal propagation after the ligand binds. This warrants additional studies to be conducted to solve the puzzle of the glycine receptor structure, adding insight into the mechanisms by which the cys-loop superfamily of receptors function.

Changes in specific amino acids of glycine receptors can render them more or less sensitive to glycine. There are many naturally occurring mutations at GlyR that cause serious and potentially fatal disorders. Substitutions of glutamine for arginine at residue 271 (R271Q; Rajendra et al., 1995) or glutamate for lysine at position 276 (K276E;

Lewis et al., 1998) for example, are two mutations that can cause hereditary startle disease, or hyperekplexia. These mutations result in a significant decrease in GlyR agonist sensitivity. Such genetic defects cause abnormal, exaggerated reflexes in response to sudden, external stimuli (Harvey et al., 2008). Loss of the glycine transporter 2 (GlyT2), which reduces glycine output, also unsurprisingly, produces the hyperekplexia phenotype (Rees et al., 2006). Many other amino acid substitutions of GlyR subunits have been found to occur in families affected by this neurological disorder. In the most recent collaborative study, various new mutants have been uncovered and subsequently studied. In detail, Chung et al. (2010) found that R65W/L, R252C and G254D mutations all resulted in either nonfunctional receptors or receptors that could not assemble properly, exhibiting no measurable currents at up to 30 mM glycine concentrations. Furthermore, E103K, S231N that were characterized by the wildtype-like maximally inducible currents had significantly higher glycine EC_{50s}. Another mutation mentioned in their studies, D165G reduces the magnitudes of currents elicited by maximally effective glycine concentrations, causing in this way a hyperekplexia phenotype (Chung et al., 2010).

Other roles that glycine receptors play are noteworthy and have been extensively described, in particular, work highlighting the importance of glycinergic signaling in amacrine cells (Wassle et al., 1986, Wassle and Boycott, 1991) and immunohistochemical research that identified Gly and $GABA_A$Rs as sources of inhibitory input of specialized cells within the mammalian retina (Grunert et al., 2000).

Considering the importance of the spinal cord in pain signal transduction it did not come as a surprise when GlyRs were found to play a role in controlling nociception. Plenty of evidence for this has been accumulated in the last couple of years. GlyR α3 subunits are found in the dorsal horn (specifically as part of lamine II) of the spinal cord where afferent endings are located. In addition, mice lacking this subunit display insensitivity to chronic inflammatory pain (Lynch et al., 2006); it should therefore prove useful to develop analgesics that specifically target this GlyR isoform.

Although it was originally believed that the β subunit plays exclusively structural and organizational roles, such as receptor localization through interaction with gephyrin (Meyer et al., 1995), recently published data proved this assumption to be wrong. Mutations studied in the β subunit established its significant role in agonist binding at interfaces between the subunits (Grudzinska et al., 2005). More comprehensive knowledge about the structure of these receptors will increase our understanding of their functioning as well as the functioning of related receptors. This will further assist us in explaining how different pharmacological agents modulate the activity of this group of CNS targets and hopefully allow us to design superior, highly selective therapeutic agents.

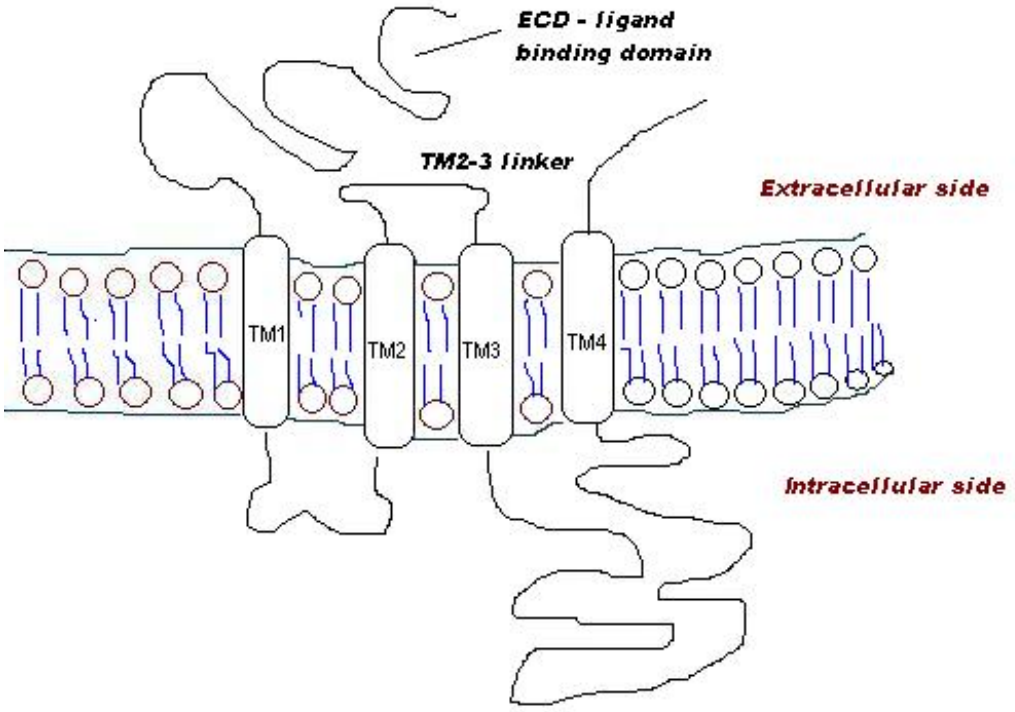


Figure 1.2 Graphical representation of a GlyR subunit A large extracellular domain where residues responsible for ligand binding are found is depicted here, together with the four transmembrane domains (TM1-4), the TM2-3 linker region (critical for signal transduction), a long intracellular TM3-4 loop and a short C terminal tail located on the extracellular side.

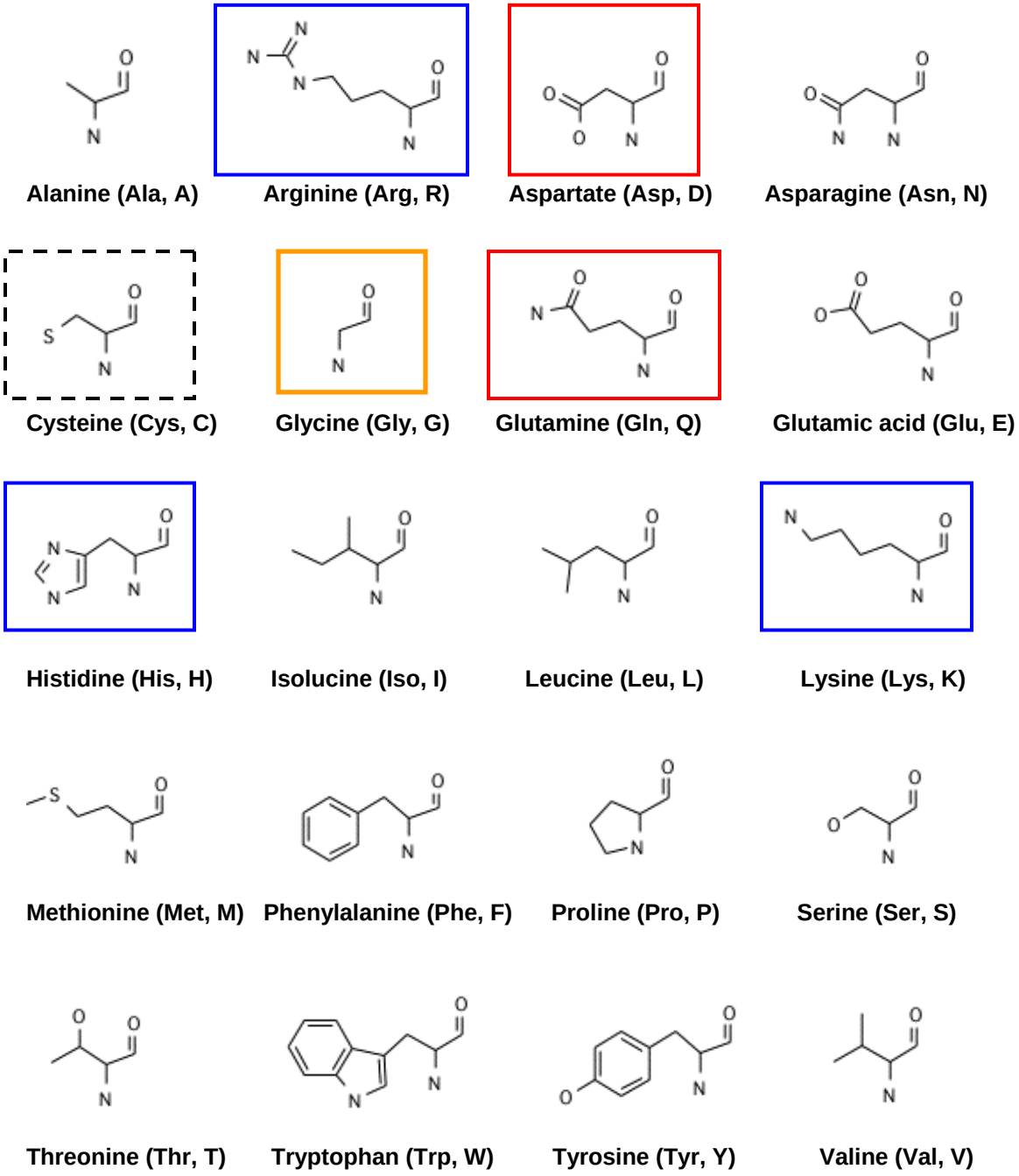


Figure 1.3 Structures and nomenclature of the twenty naturally-occurring amino acids Glycine is boxed in orange, negatively charged residues in red, and those carrying an overall positive charge in blue. A black dashed box highlights cysteine, a residue capable of forming a disulfide bridge. (*Adapted from Timothy Paustian, http://lecturer.ukdw.ac.id/dhira/BacterialStructure/Proteins.html, 2001*)

1.3 Agonist binding properties

In order for the intrinsic channel of GlyR to open, the receptor has to be activatable and, in most cases, the agonist has to be present. This activatable (but not yet ion permeating) state is called the closed state, and transitions among states are simplistically outlined below:

$$\mathbf{RC + A} \rightleftharpoons \mathbf{RAC} \rightleftharpoons \mathbf{RAO}$$

Here, RC represents a receptor found in its most stable closed state, A is the agonist, RAC is a receptor going through conformational changes caused by agonist binding, and RAO is a receptor conducting ions through the open pore.

When glycine binds to initiate channel opening, it does so by interacting with specific amino acids located at the interfaces between the subunits. Six regions of amino acids constitute the known binding site for glycine on the GlyR. On the plus (+) side of the interface, between the adjacent subunits, loops A, B and C come together and interact with β sheets labeled D, E, F carrying residues that form the opposite, minus (–) side of the binding region (Lynch, 2004). Many different groups have worked on building the knowledge that we today have about the location of glycine binding. The work on identifying the exact residues that may interact with glycine has proven difficult, but nonetheless, informative. We now know that loop A contributes residues I93 and N102 (Vafa et al., 1999). It has also been shown that residues R119, as well as R131, play a significant role in the formation of the binding pocket. Domain B mutants have been harder to interpret, since all of those studied seemed to affect both binding and gating aspects of glycine receptor activation. Nevertheless, available data point to the

importance of the F159, G160 and Y161 residues (Schmieden et al., 1993). Loop C is interesting because it is a part of a second cys-loop, found only in the GlyR. This disulfide bridge appears to play a role in stabilizing the secondary structure of the agonist binding domain by keeping the residues important for interacting with the agonist in the proper orientation. Residues from C loop, shown to have a significant role in agonist binding, are L200, Y202 and T204 (Rajendra et al., 1995). A complete understanding of the complementary (-) side of the binding interface, strands D, E and F, still remains elusive and work on this region will help us further our understanding of the structure of the binding pocket as well as strengthen what we know about the mechanism of binding. An aspartate residue at position 97 (D97) was implicated previously (Beckstead et al., 2002) as an important part of the (+) side of the binding interface. When this aspartate is mutated to any other amino acid this renders GlyR spontaneously active, showing a critical role of this amino acid for closed state stability and receptor activation (Chapter 3: Todorovic et al., 2010). Besides the role it plays in stabilizing the receptor in the closed state it also affects glycine EC_{50} and strychnine IC_{50} values. This strongly suggests a role for this residue in the formation of the agonist-binding pocket.

Until recently it was believed that the α subunit alone contributes to the glycine binding domain. This thought arose from the finding that incorporating a β subunit reduces apparent cooperativity of binding (represented with the lower Hill coefficient value). However in 2005, a paper published in Neuron showed that agonist efficacy is indeed affected by β subunit mutations (Grudzinska et al., 2005). In particular, this group found that negatively charged glutamic acid residues at positions 157 and 180 on the β

subunit appear to interact with the glycine molecule, by possibly stabilizing its α amino group (Grudzinska et al., 2005). They also note that, as part of the (-) interface, positively charged arginines at positions 65 and 86 contribute to glycine binding as well, most likely by interacting with the glycine α carboxylate side chain (Grudzinska et al., 2005).

1.4 Signal transduction connects agonist binding and channel gating

After a glycine molecule docks at the interface between the subunits this binding signal has to travel more than 40 angstroms to the pore region in order for the channel to be opened (Miyazawa et al., 1999).

The mechanism responsible for this activation has been and continues to be a major mystery, and knowledge of the steps involved in the activation pathway of GlyR would explain how different pharmacologically important agents such as ethanol modulate this process. More detailed knowledge of this mechanism would then allow us to find more selective agents that would be able to modulate different steps of glycine receptor function. In addition, the details of the movements required for activation would also add to our understanding of receptor structure.

In the related nAChR, signal transduction after agonist binding has been described as a "Brownian conformational wave" that travels down the interface between the binding subunits (Purohit and Auerbach, 2007). Auerbach's group performed calculations to show time-dependent relative movements of different extracellular segments of the nAChR, that they label "Phi blocks". Their calculations are based on the ratio between the rates of activation (β) and rates of deactivation (α) measured using multiple mutant

receptors, each representative of a different segment. They call the calculated ratio values ϕ, representing the relationship between free energy changes that occur as the receptor switches between the "end" states (open and closed). As expected, the binding domain/loop 5 (Chakrapani et al., 2003) of the muscle nAChR has the highest ϕ value (0.93), which translates into an almost instant movement after acetylcholine binding. Of all the mutants they made nAChR αD97 showed the highest phi scores, implicating this area in moving early in the activation process. Movement of loops 2 and 7 comes next (Chakrapani et al., 2004, Purohit et al., 2007), followed by the M2 cap, a segment homologous to GlyR's TM2-3 linker region. This area has been implicated in significant detail in channel activation (sitting about midway between the binding site and the gate) in many members of cys-loop receptor superfamily of subunits (Absalom et al., 2003, Bafna et al., 2008). Although similar work as the ϕ analyses described by Auerbach's group for the nAChR is currently unavailable for GlyRs, many studies have looked at comparable domains in an attempt to shed some light on allosteric transduction process after glycine initiates opening.

Extensive studies on pre TM1, TM1-2 loop and the TM2-3 linker region have uncovered the importance that these segments play in proper channel activation (Lynch et al., 1997). It is not surprising that residues close to the pore-forming TM2 domain constitute an important transduction point for the binding signal. Therefore, most likely due to the proximity of these residues to the pore, almost any change in these segments will cause a disruption of signal transduction to the gate. Kash et al. (2003) illustrated the importance of the interaction between loops 2 and 7 (the disulfide loop conserved in all

cys-loop receptors) of the $GABA_AR$ with the TM2-3 linker region in bridging binding and gating processes. The Schofield group revisited this interaction in GlyR, hypothesizing that during channel activation, charged residues, located in these loops, are also necessary for proper activation of GlyRs (Schofield et al., 2003). They concluded that residues in loop 2 and loop 7 (conserved cys loop) play an important role in the activation of GlyR but do not relay the message to the base of ECD/pore mouth by direct electrostatic interaction with residues from the TM2-3 loop as seen in $GABA_AR$ study (Schofield et al., 2003). This points to differences among activation processes in different members of the cys-loop family of receptor-activated channels.

Additional evidence for the importance of the TM2-3 linker region in transducing the binding message came from a detailed study of the conserved arginine residue at position 271 in the α1 GlyR subunit. Mutations of this residue occur naturally and are known to affect glycine efficacy and potency as well as decrease ion conductance across the pore (Langosch et. al., 1994). As mentioned above R271L and R271Q are mutant receptors that cause hyperekplexia (Ryan et al., 1992). This mutation also converts taurine and β alanine into competitive antagonists while not affecting their binding profiles (Laube et al., 1995). All this points to the importance of this residue, and this region in general, in the proper activation and gating of GlyR. Mutations of comparable residues in other cys-loop LGICs also affect gating and as a result cause various disorders (Croxen et al., 1997, Baulac et al., 2001).

The cysteine accessibility method was used previously to study the residues of the TM2-TM3 linker region. This work confirmed that changes in these residues disrupt the

activation process and has also uncovered higher than expected flexibility of the region. (Dupre et al., in preparation). Specifically, many of the single cysteine mutants in this area show crosslinking which, when assayed in C41A and C290A mutant backgrounds have no conceivable way of forming the disulfide bonds other than between the subunits. This indicates that the TM2-3 linker has a high degree of flexibility, a characteristic that one would expect in a region implicated in interaction with the binding domain.

1.5 Gating and ion permeation

Residues of the TM2 segment line the GlyR pore and form the gate that is ultimately responsible for ion permeation. The gate forms the barrier dividing the extracellular and intracellular milieu. It is at the gate that many functional decisions are made, namely, what ions pass (ion selectivity), when ions pass through (conducting and non-conducting receptor states) and how many ions pass per unit time (unitary conductance). The GlyR pore is lined with hydrophilic residues and it contains two rings of positive charges, R252 and R271 that are believed to attract and organize anions before they are transported across the membrane (Keramidas et al., 2004). Substitution of arginine 252 by alanine prevents the expression of the receptors so this position could not be studied in detail. When residues around this site are mutated this causes a reversal in permeability, causing the GlyR channel to conduct cations. This most likely occurs when positively charged arginine side chains at position 252 are not positioned properly, changing in this way ion selectivity. Mutations of R271 do not change ion selectivity even when negatively charged residues are engineered at this position, but, as mentioned

previously, naturally-occurring mutations at this position do in fact affect channel function and cause hereditary startle disease (Shiang et al., 1993). Data from Bormann et al. (1994) support the role of this residue in affecting conductance. When the β subunit is co-expressed with an α subunit the conductance is about half that of homomeric α receptors. It is interesting to note that when a negatively charged glutamic acid found at the position 270 of the β subunit (the position homologous to α1 R271) is substituted with serine, the resulting heteromeric αβ(E270S) GlyRs display increased conductance, similar to the levels recorded from α1 homomeric receptors.

GlyR single channel recordings report variable unitary conductance. This depends on the expression system and subunit composition. The most frequently measured heteromeric αβ GlyR conducting states reported in 42-50 pS range with lower subconductance states between 29-36 pS (Bormann et al., 1993). The wild type glycine receptor is permeable to many anions and the order of ion permeability is outlined below:

$$\mathbf{SCN^- > NO_3^- > I^- > Br^- > Cl^- > F^-}$$

Although GlyR can permit different ions to pass across the pore, *in vivo*, Cl^- is the most abundant anion in both the intracellular and extracellular environment, and therefore the primary anion that this channel will conduct. When this occurs, the membrane potential will be pushed towards the Cl^- equilibrium potential which will in turn change the excitability potential of the postsynaptic cell.

1.6 Single channel properties

On a whole cell level, electrophysiological recordings are limited in many ways. Data collected does not allow us to individually assay many of the intrinsic properties of the receptor, preventing a fuller understanding of receptor function. Whole cell recordings involve the simultaneous recording of millions of receptors and results are influenced by a number of unknowns: expression levels, desensitization rates and possibly receptor-receptor interactions. Employing patch clamp electrophysiology can successfully avoid some of these problems. In 1991, the Nobel Prize for Physiology and Medicine was awarded to Bert Sakmann and Erwin Neher for their groundbreaking advancement in ion channel research. They realized that by using a glass pipette tip with an extremely small diameter they could make a tight contact with the cell from which they were trying to record. The contact between the pipette and the membrane made a high-resistance gigaOhm seal, decreasing the background noise and making small, picoamp single channel current recordings a possibility. The advent of this new method and subsequent years of improvement led to significant progress in ion channel research. Hamill and Sakmann (1981) reviewed single channel conductance states in acetylcholine receptors and soon after, the same group of scientists published the first paper describing GlyR single channel properties. In their 1983 Nature paper, Hamill et al. report that unitary conductance levels, recorded from mouse spinal neurons, in the presence of GABA or glycine differed, depending on agonist used. This was the first strong evidence in support of the hypothesis that these two endogenous agonists activated different receptors (Hamill et al., 1983). The same year, they published additional data, using the

cell-attached patch clamp protocol, on glycine and GABA receptors from spinal cord neurons, showing that receptors open after the two agonists have bound and going on to describe what were just the beginning insights into glycine receptor function (Sakmann et al., 1983). More detailed studies on GlyR single channel kinetics came a couple of years later when Twyman and Macdonald (1991) published their work on glycine receptor currents measured from mouse spinal cord neurons *in vitro*. From their recordings they concluded that the GlyR has three distinct open state conformations: short-, medium- and long-lived states. They also found that as the concentration of glycine increased so did the frequency of occurrence of the longer-lived states. This, they thought, indicated that longer-lived states were probably characterized by the receptor being occupied by more ligand molecules (more often) than at lower concentrations (Twyman and Macdonald, 1991). Single channel recording techniques and analysis kept improving and the following years brought more insights into the kinetics of GlyR function. Beato and colleagues (2004), for example, studied GlyR single channel kinetics at maximally-effective glycine concentrations (1mM) and showed that when the receptor is saturated with agonist, it primarily opens to only the longest-lived open state. By testing different models to explain their data, they demonstrated that at saturating glycine concentrations, the best fit of the data could be explained by a model involving the binding of three agonist molecules; adding a fourth or fifth binding occurrence did not improve the overall fit of the data, and that if these additional binding events occurred, it did not significantly change the efficacy of channel gating (Beato et al., 2004). The same group repeated comparable recordings on heteromeric $\alpha 1\beta$ receptors and arrived at the same

conclusions; as glycine concentration increased so did the probability of channel opening (Beato et al., 2007). Again, at higher glycine concentrations the channels increasingly opened to the longest-lived state. Heteromeric $\alpha 1\beta$ glycine receptor activation was fit best with up to three occupied binding sites, equal to the number of binding events used to fit data from the homomeric receptors. Just recently another important component was hypothesized to be necessary for the better fit of single channel data. These additional closed states, termed "flipped", are proposed to be intermediates representing the transition of the channel between the closed and open states. First introduced by the Sivilotti and Colquhoun group, this flip mechanism is suggested to represent the channel in a ligand-bound "closed, but high affinity state" (Lape et al., 2008). A model, which includes this "pre-open" conformation, has already been used in an attempt to explain the mechanism of partial agonist activation on cys-loop receptors. Both Sivilotti and others (Welsh et al., in revision) propose that partial agonists at GlyR cannot achieve full efficacy because of their lower rate of transitioning the receptor from the closed (bound/resting) to the flipped state; as was shown previously partial and full agonists have almost identical kinetics of transitioning to and from the open state from this intermediate, flipped state (Sivilotti, 2010).

Single channel electrophysiology has also allowed for a more precise understanding of allosteric modulation of GlyR function. The application of ethanol with glycine was shown to increase mean open burst durations (Welsh et al., 2009) while another group demonstrated that potentiating concentrations of Zn^{2+} affect GlyR kinetics

by increasing the probability of channel opening while also increasing burst durations (Laube et al., 2000). Neither ethanol nor Zn^{2+} affect GlyR conductance.

Although single channel recordings obviate many weaknesses of macroscopic recordings, much needs to improve in order to perfect this method and to fully comprehend the function of the channels from which we are recording. Missed events and the choice of appropriate models for data fits are some of the areas where more work is necessary if we are to continue to make steps forward in fully appreciating the function of ion channels.

1.7 Partial agonists

Ligand gated ion channels all have more than one ligand capable of gating them, at varying efficacies. Until recently there was no clue as to why glycine acts as a full agonist while taurine, β-alanine, and even GABA can all bind at GlyR but are, in most cases, all characterized by possessing lesser efficacy. If co-applied with the full agonist, a partial agonist will exert antagonistic properties, competing for receptor occupancy, decreasing in this way the efficacy of the full agonist. A review of taurine, one partial agonist at the GlyR, is included below.

Taurine acts mainly as a partial agonist on GlyR although its efficacy can vary across different expression systems. This β amino acid is found in many different organs of mammals, with the highest concentration found in intestines. It was first isolated from ox bile, and appropriately named taurine from the Latin, *Taurus* meaning bull. It can exist in cis and trans forms, an important characteristic for its binding to various target

proteins. In its cis structure taurine is like glycine while the trans form resembles the competitive GlyR antagonist nipecotic acid (**see Structures of Compounds - xxiii**). Whether this cis/trans isomerization determines the efficacy of taurine at the GlyR remains to be seen. In the R271Q and R271L mutations that cause hyperekplexia, taurine acts as an antagonist (Laube et al., 1995). Other residues such as E53 and E57 are significant determinants of taurine binding since mutations at these positions also change taurine into an antagonist at the GlyR (Absalom et al., 2003). A mutation of the ECD residue D97 that renders GlyRs spontaneously active increases the efficacy of taurine, making it a full agonist at these receptors (Welsh et al., in revision). Other mutations also change the efficacy of this endogenous compound. One such mutation, I244N, located on the intracellular side of TM2, causes a decrease in taurine's efficacy while also changing receptor desensitization rates (Lynch et al., 1997). Taurine's efficacy on GlyR also varies between different expression systems. For example, it acts as almost a full agonist when GlyRs are expressed in HEK293 cells but displays about 45% of the efficacy of glycine on receptors expressed in *Xenopus* oocytes (Farroni and McCool, 2004). A more dramatic decrease in taurine's efficacy was seen in GlyRs expressed in L-cell fibroblasts (Farroni and McCool, 2004). In addition, differences in agonist efficacies also exist between α homomeric and $\alpha\beta$ heteromeric receptors.

A number of studies have investigated how taurine and glycine signal transduction compare. The ability of thiosulfonate agents to bind residues of the TM2-3 linker region was studied. This segment was previously shown to play a vital role in the proper activation of GlyRs (Lynch et al., 2001, Absalom et al., 2003). Han et al. (2004)

demonstrated that changes that occur at the TM2-3 linker region as the result of receptor activation by glycine and taurine are comparable. Therefore they concluded that taurine efficacy must depend on the ability to stabilize the open state and not by the steps preceding the opening of the channel (Han et al., 2004)

1.8 Antagonists

An antagonist can be defined as any compound that prevents an agonist from exerting its effect, but does not itself possess efficacy. These compounds either compete for the same or overlapping binding sites with agonists, or block the activity in an allosteric way (channel blockers). Among the different antagonists that act on GlyRs, strychnine and picrotoxin (PTX) are the two used in the dissertation research described below (**Structures of relevant GlyR ligands – xxii**).

a) Strychnine

Seeds of the *Strychnos nux vomica* plant are the largest known source for the GlyR competitive antagonist strychnine. Although this plant alkaloid was once prescribed for different ailments and even used as a stimulant by athletes, today's use is limited to research and represents one of the most valuable compounds when studying inhibitory synapses and GlyRs. Strychnine and glycine binding sites are not identical but, as expected, overlap. From the work by Grudzinska et al. (2005) it is hypothesized that residues coordinating the binding of this compound are R131 and E157 on the minus side of the binding interface and Y202 and F207 on the plus side of an adjacent subunit.

Strychnine acts as a competitive antagonist by shifting the glycine concentration-response curve to the right without affecting the maximally-attainable current. The sensitivity of GlyR to strychnine depends on subunit composition. This competitive antagonist has greater affinity for α1 than α2 subunit-containing receptors (Tapia et al., 1998, Ye, 2000).

b) Picrotoxin

Another highly poisonous plant alkaloid, picrotoxin, is most often isolated from the fruit *Cocculus indicus*. Consisting of equal amounts of two almost identical compounds, picrotin and picrotoxinin, it acts as a noncompetitive antagonist on $GABA_A$Rs by blocking the channel pore (Newland and Cull-Candy, 1992, Chang and Weiss, 2002, Olsen, 2000). The mechanism of PTX action on GlyR is still somewhat unclear. While some groups argue that it acts as a pore blocker, like at the $GABA_A$R, others believe that its non-use dependence hints at a different mechanism of action on GlyR. Some of the first studies demonstrated a loss or reduction of PTX effects when 2' and 6' arginine residues within the GlyR pore, were mutated (Lynch et al., 1995). Furthermore, in a study conducted by Hawthorne and Lynch (2005), they engineered a cysteine at position R271, at the mouth of the channel, and demonstrated that PTX inhibition could only be reversed with additional glycine application. This, they felt, strengthened the evidence for PTX binding in the pore and thus acting as a pore blocker. Due to the significant difference in binding affinity PTX can be used to distinguish between homomeric and heteromeric GlyRs. The presence of the β subunit makes the

receptors significantly less sensitive to the effects of PTX. This is caused by a difference in TM2 residues between α and β subunits. This strengthens the evidence that this compound indeed binds in the pore (Pribilla et al., 1992) but without definitive proof that it actually blocks the glycine receptor pore.

1.9 Allosteric modulators

a) Alcohols and anesthetics

Alcohol is a common name for the organic compound ethanol (EtOH), present as the active ingredient of alcoholic beverages. Residuals found on excavated pottery pieces, dated to Neolithic period, imply that humans may have used alcohol since as early as 9000 BC. Since these early times, alcohol abuse and dependence have become one of the biggest problems of the modern society. With billions of dollars spent every year to treat diseases caused by alcohol abuse, and alcohol dependence itself, it is not surprising that considerable effort is being expended to improve our understanding of the development of dependence as well as symptoms of drug withdrawal. Knowing the specific molecular targets responsible for the various effects of ethanol could help explain these processes and aid the search for selective agents that could treat symptoms of the withdrawal and prevent relapse in alcoholics.

Until the last two decades it was believed that alcohols acted exclusively by nonspecifically disordering neuronal membranes. Even though considerable progress has been made in highlighting the roles of more specific targets for alcohol, the debate of

where ethanol binds to exert its many actions, still remains. This CNS depressant has been implicated in causing its behavioral effects by acting on different neuronal proteins. Not surprisingly, it has been shown to enhance $GABA_A$ and glycine receptor function, elevating in this way the inhibitory tone in the CNS.

Initial studies showed that single and double mutations of receptor subunits can prevent alcohol potentiation of GlyR and $GABA_AR$ function. The Harris group first demonstrated that a mutation responsible for the spasmodic phenotype in mice (α1A52S), results in a decreased effect of ethanol on GlyR when compared to the potentiation seen in the wt α1GlyR (Mascia et al., 1996). The site of alcohol action on these receptors was localized using various chimeric constructs consisting of different segments of the α1GlyR and the related $GABA_C$ receptor whose function is inhibited by alcohols and anesthetics. These findings revealed the TM2 and TM3 segments that are critical for observing the potentiating actions of alcohols and volatile anesthetics at GlyR (Mihic et al., 1997). More importantly, they identified two residues that when mutated either decreased or completely abolished the enhancing actions of these compounds. In GlyR, serine at position 267 of the TM2 domain and alanine at position 288 of the third TM region were the two residues initially implicated in forming the alcohol and anesthetic binding pocket (Mihic et al., 1997).

Since those initial studies, many experiments have been conducted studying these and other residues to verify the existence of the alcohol and volatile anesthetic binding domain and its conservation throughout the LGIC family. In series of studies Ingrid showed that in α1 GlyR S267 (TM2), A288 (TM3) and another TM residue isoleucine

293 of TM4 can crosslink within the subunit, postulating that in this way they form a cavity where various allosteric modulators can fit, exerting in this way their actions at these receptors (Lobo et al., 2004a, Lobo et al., 2008). In her studies using MTS reagents, she also showed that this pocket, formed between the membrane domains, is water accessible (Lobo et al., 2004b, Lobo et al., 2006). A study investigating the region postulated to form an alcohol and anesthetic binding pocket used cysteine labeling and disulfide trapping to determine whether residues S267 (TM2) and A288 (TM3) played a direct role in the binding of alcohols and anesthetics (Mascia et al., 2000). They investigated whether propanethiol, an anesthetic with a structure comparable to that of the alcohol propanol, would be able to form a covalent bond with C267 and/or C288 engineered residues. Their results allowed them to conclude that position 267 of the TM2 domain can form a direct bond with both propanethiol and PMTS (another thiol reagent). Binding of both of these thiol reagents resulted in irreversible potentiation of α1(S267C) GlyR function. This strengthens the hypothesis that this is the most likely site for direct binding of alcohols and anesthetics. They did not observe the same results on the A288 residue even though this position was postulated in previous studies to be in direct contact with S267 (Mascia et al., 2000).

Before these *in vitro* studies (reviewed above) had been conducted, several behavioral studies gave some convincing evidence for a site of ethanol action at the GlyR. In particular, a study in mice showed that glycine and the glycine precursor, serine, could positively affect ethanol action. In addition, strychnine abolished these effects, strongly implying that the action was controlled by strychnine-sensitive GlyRs (Williams

et al., 1995). On the behavioral level, studies have also demonstrated that knock-in mice carrying the S267Q α1 mutation rendered animals less sensitive to the effects of ethanol, seen as an improvement of animal motor coordination in the presence of ethanol (Findlay et al., 2002).

b) Zinc (Zn^{2+})

After iron, Zn^{2+} is a second most abundant transition metal found in mammalian bodies. This divalent cation affects many biological processes: it is necessary for various enzymatic reactions, RNA and DNA metabolism, and is vital for many aspects of CNS function and modulation. In neurons, Zn^{2+} is stored in presynaptic vesicles and released onto postsynaptic membranes with neurotransmitters. In GlyRs Zn^{2+} has potentiating effects at low concentrations (low nM - 10μM) and an inhibitory effect at concentrations exceeding 10μM. Studies related to the general binding properties of Zn^{2+} have shown that the most likely residues to coordinate the binding of Zn^{2+} would be histidine residues. Site directed mutagenesis experiments demonstrated that two histidines in the ECD, H107 and H109, regulate the inhibitory actions of Zn^{2+} on GlyR (Harvey et al., 2001, Nevin et al., 2003). Nevin et al. (2003) provide strong evidence that the binding of Zn^{2+} to this site occurs at the interface of two adjacent subunits. From their work on heteromeric α1βGlyRs they concluded that only the α-α interface is crucial for the modulation of channel activity by Zn^{2+}. On the single channel level it was shown that, at inhibitory concentrations, Zn^{2+} decreases the amount of time channels remain open by

stabilizing the closed state and decreasing the gating efficacy of the receptor (Laube et al., 2000).

The high affinity, potentiating, site of Zn^{2+} binding has, for the longest time, remained quite elusive. The first studies that uncovered the region critical for the potentiating effects of Zn^{2+} were conducted on homomeric α1GlyR. When aspartic acid at position 80 of the ECD was replaced by alanine (α1D80A) this prevented Zn^{2+} enhancement of glycine currents, but did otherwise not affect receptor function (Lynch et al., 1998, Laube et al., 2000). The significant role of this residue for Zn^{2+} action was proved further when Hirzel et al. (2006) demonstrated that mice homozygous for this mutation (Glra1 D80A) developed a hyperekplexia-like disorder. Electrophysiological data from obtained from hypoglossal brainstem slices of the same animals demonstrated no other significant differences in glycinergic organization or transmission (Hirzel et al., 2006). Other residues were also implicated in playing a role in the potentiating effects of Zn^{2+}. In particular, residues α1E192, α1D194 and α1H215 have all been proposed to either stabilize Zn^{2+} binding or have a role in transducing the signal following the zinc binding event (Miller et al., 2005).

At a concentration of 10μM, Zn2+ was found to increase the efficacy of the partial agonist taurine. In the same study it was reported that on the single channel level Zn^{2+} increased the open channel probability at a low (5μM) zinc concentration by decreasing the rate at which agonist dissociates once bound (Laube et al., 2000).

1.10 Statement of purpose

The work presented in this dissertation attempts to further our knowledge of GlyR structure and function. Our data obtained using α1 homomeric GlyRs demonstrated that the charged residue aspartate at position 97 (α1D97) in the extracellular domain plays an important role in the regulation of channel opening and closing events. In this dissertation I determined the role that this residue plays in channel activation. It is accepted today that there are many important interactions among residues of GlyR subunits that play critical roles in all aspects of receptor function, including but not limited to the receptor stability in its different states, signal transduction, ion permeability and channel activation.

The data presented here involves the study of a number of different interactions that we believe control some of the above-mentioned GlyR features.

Aim 1: Identify the salt bridge residues necessary to restore closed-channel stability to the spontaneously opening GlyR

We previously determined that any mutation of the aspartic acid residue at position 97 (α1D97), located in the ECD destabilizes receptors and renders them tonically active in the absence of the agonist. From initial modeling studies in which the glycine receptor sequence was threaded on both the known structures of the nACh binding protein (nAChBP) and *Torpedo* nAChR, two different residues, a positively charged lysine at position 116 and an arginine 119, were postulated to be at the right

position to interact with α1D97. It was hypothesized that re-establishing this electrostatic bond by a second mutation would stabilize the closed state of the receptor in the absence of an agonist, and in doing so decrease or abolish the spontaneous activity recorded in all of the single α1D97 mutants.

Aim 2: Determine that the salt bridge controlling GlyR channel gating occurs between adjacent subunits

We hypothesized that α1D97 interacts with the positively charged residue R119 located on an adjacent subunit. The purpose of this aim was to gain more information about the nature of the D97-R119 salt bridge that regulates channel opening and closing events. The basic question addressed in this aim was whether the α1D97 and α1R119 electrostatic interaction occurs between subunits or within the subunit. To differentiate between these two possibilities we co-injected single mutant cDNAs coding for either the α1D97C or α1R119C subunits, in a ratio that favored expression of the α1R119C subunit. We then functionally identified receptors carrying both α1D97C and α1R119C subunits using disulfide trapping. The existence of cross-linking in that scenario would allow us to conclude with certainty that the D97-R119 electrostatic interaction between these charged residues occurs between adjacent subunits.

Aim 3: Identify other residues that are interacting with residue D97

From the structure of the prokaryotic cys-loop channel GLIC, a new model was devised that included another charged residue as a potential amino acid that could play a role in interacting with α1D97 to keep the receptor in a stable closed state. This residue, α1R131, has previously been implicated in many other aspects of GlyR function, making it an interesting target to investigate. In particular, a recent study (Laube et al., 2002) implicates this residue in both glycine and strychnine binding stabilization. This characteristic would be expected from a residue that may contribute to a cloud of charges that regulates the structure of the binding interface and stability of the closed state before binding is initiated. We hypothesized that interaction occurs between α1D97 and α1R131 and tested this by performing electrophysiological studies on the α1D97R/R131D double mutant like we had previously done on the α1D97R/R119D double mutant in Aim 1. We also tested for disulfide bond formation between these positions when residues 97 and 131 were substituted with cysteines.

Aim 4: Characterize the structure of the extracellular portion of TM2 responsible for signal transduction

Binding of glycine in the ECD between adjacent subunits is the first step in the activation of GlyR. The binding signal must then ultimately be transduced to the pore and a number of distinct regions of these receptor subunits have been implicated in this transduction process. One of these regions is the extracellular portion of TM2 and the

TM2-3 linker region. Discrepancies in results among labs have brought on a conflict of thoughts on what the more extracellular segment of TM2 (residues T265 to S270) looks like structurally. Unwin's 4.6 angstrom model of the nAChR reports this area as a constrained true α helix (Unwin et al., 2005). However, Dejian Ma and group published conflicting data in their work on the GlyR, concluding that this region, at least in this anionic receptor, is characterized by more relaxed structure and a greater degree of flexibility (Ma et al., 2005). This structural feature may play a significant role in allosteric activation and modulation of LGIC's and could further explain key elements of glycine signal transduction and the kinetics of gating. We employed cysteine mutagenesis and disulfide trapping in order to test this electrophysiologically. True α helical structure in this region would be expected to allow every third or fourth residue in a segment to be accessible and to point in same direction. If this stretch of amino acids is indeed constrained in a true α helical fashion only a few of the residues should be able to interact with equivalent residues on an adjacent subunit, but if there is less restriction in their movement this could occur in more (or all) of the proposed residues.

CHAPTER 2: EXPERIMENTAL PROCEDURES

2.1 Primer design

Primers were custom-designed and supplied by Integrated DNA Technologies (Coralville, IA). To make primers we designed 35-45 base length sequences, with more than 60% GC content, possessing a melting point (T_m) greater than 78^o, and a minimal possibility for intraplasmid loop formation (as outlined in the book Methods in Alcohol-Related Neuroscience Research, Liu Y. and Lovinger D. M., 2000). Standard desalting and HPLC purification procedures were utilized to ensure the purity of samples.

2.2 Site directed mutagenesis

The human α1 GlyR subunit cDNA was subcloned into a modified pBK-CMV vector lacking the *lac* promoter and the *lacZ* ATG (Mihic et al., 1997). All mutant receptors were made using this plasmid except for S267 mutations, which were subcloned into the pCIS plasmid vector. Both of these plasmids contain the CMV promoter that allows them to be expressed in both HEK293 cells and *Xenopus* oocytes. One difference between the two vectors is that the pBKCMV vector contains a kanamycin resistance gene while pCIS2 contains a gene conferring ampicillin resistance. Antibiotics were introduced into the LB (Luria Broth) agar plates that were used for the overnight growth (at 37^o) of XL-1 supercompetent bacterial cells carrying the vector with the desired cDNA construct. LB media used to grow separate selected colonies was also

enriched with the appropriate antibiotic, at a concentration of 50 μg/ml for kanamycin and 100 μg/ml for ampicillin. Sterile tubes with loose tops, 3ml of Luria broth, appropriate antibiotic and chosen colonies were placed in a shaker and incubated at 37°C. To prevent accumulation of bacterial growth byproducts, the incubation time was limited to 18 hours. A miniprep kit was used to isolate the plasmid and gather the cDNA of interest, as described in the provided protocol (Invitrogen). The Stratagene (Cedar Creek, TX) QuickChange Mutagenesis Kit was used to create all mutations. Each mutation was constructed by using the appropriate template (in our case either α1 or β GlyR sequence at a concentration of 25 ng/μL), plasmid primers (125 ng/μL), nucleotide tri phosphates (NTPs), and Taq polymerase. The PTC-100 termocycler was used for linear amplification: template cDNA was denatured at 95°, the temperature then lowered to 55° to allow for annealing of the primer sequence and finally raised to 68° to allow for extension at the 3' end to form the product of the reaction. This is set to repeat 30 times. After cycling the product was incubated with the enzyme *Dpn I* that cleaves the methylated template, leaving intact only the newly formed mutated version. Transformation then occurred as described above and the accuracy of the introduced mutation was verified by the sequencing facility at the University of Texas at Austin. To ensure cleanliness and to determine the concentration of our samples they were analyzed using the ND-1000 spectrophotometer. Acceptable quality of cDNA requires a $A_{260/280}$ ratio greater than 1.8 and only samples that passed this quality control were kept as stocks for dilutions (when necessary) and injection.

2.3 *Xenopus leavis* oocyte isolation and cDNA injection

Oocytes were surgically removed from *Xenopus leavis* in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Oocytes were first held in isolation media, containing in mM: 108 NaCl, 10 HEPES, 2 KCl and 1 EDTA. This allows for partial dehydration of the oocyte, which will allow for easier removal of the thecal and epithelial membrane layers. After manual isolation oocytes are kept for 10 minutes in a collagenase solution (mM: 83 NaCl, 5 HEPES and 2 $MgCl_2$ with 5 mg/10ml collagenase D enzyme) in order to remove the follicular layers. Oocytes are then stored in sterile Modified Barth's Saline (MBS) and nuclear cDNA injections, at concentrations ranging from 0.03-3 ng/30nl, are performed. MBS contains (in mM): 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 10 HEPES, 0.82 $MgSO_4 \cdot 7H_2O$, 0.33 $Ca(NO_3)_2$ and 0.91 $CaCl_2$ at pH 7.5. In order to ensure that the cDNA will reach the nucleus injections are delivered to the center of the oocyte animal pole, approximately one quarter of the diameter into the oocyte. After injection oocytes are separated and kept individually in sterile 96 well plates (Costar) in incubation media (1X MBS enriched with 2 mM Na pyruvate, 0.5 mM theophylline, 10 U/ml penicillin, 10 mg/l streptomycin and 50 mg gentamycin and sterilized by filtration using 0.22 μm filter). Plates are placed in a room at a controlled temperature (≈ 19^o C) and held in the dark until ready for experiments (usually between 1-10 days).

2.4 Whole-cell oocyte electrophysiological recordings

All whole-cell experiments were conducted at room temperature (22-24^{o} C) with oocytes voltage-clamped at a holding potential of -70mV using a Warner Instrument OC725C (Hamden, CT) oocyte clamp. Oocytes were perfused with MBS at a rate of 2 ml/min through 18-gauge polyethylene tubing (Benton Dickinson, Sparks, MD) using a peristaltic pump from Cole Parmer Instruments (Vernon Hills, IL). Currents were recorded using a Cole Parmer Instruments chart recorder from the same company. All of the compounds used to produce or modify currents were dissolved in MBS prior to application. Complete concentration-response curves were determined where possible, by applying 3-60 sec pulses of glycine separated by 10-15 min washouts between applications. In experiments in which we measured spontaneous activity of mutant receptors, 1-10 mM strychnine was applied for 60 seconds. During application of cross-linking, oxidizing or reducing reagents oocytes were unclamped and electrodes removed from the bath to minimize their exposures to these agents.

2.5 Patch clamp electrophysiology, acquisition and analysis

Outside-out patches recordings were made according to standard methods (Hamill et al., 1981) and those methods are described in Welsh et al. (2009). Single channel data were analyzed as described previously (Welsh et al. 2009) using the single channel analysis programs in QuB (Qin et al., 2000a, Qin et al., 2000b) (version 1.4.0.125). A Star model incorporating two open and two closed states was used for idealization and fitting. Individual clusters were identified in the wildtype and D97R mutant GlyR by

eye. For each cluster the probability of channels being found in the open state (P_{open}) was determined by dividing the open time in the cluster by the cluster length. In the D97R/R119E double mutant, defined clusters could not be delineated; the P_{open} in this case was determined by sampling several portions of the tracings that did not contain multiple simultaneous openings.

2.6 Modeling

A homology model of GlyR α1 was built by threading the GlyR primary sequence onto an X-ray crystal structure template using the Modeler module of Discovery Studio (DS 2.1; Accelrys, Inc, San Diego, CA) as previously described (Crawford et al., 2007). The template we used was the prokaryotic ligand-gated ion channel homologue GLIC (PDB ID 3EAM) (Bocquet et al., 2009) in part because the X-ray structure of GLIC has higher resolution than the previous cryo-electron microscopy structure of torpedo nAChR (PDB ID 2BG9). In addition, GLIC has higher homology to GlyR than to nAChR. However, note that the sequence of GLIC does not contain the N-terminal alpha helix of the eukaryotic Cys-loop receptors; the alignment of the GlyR sequence starts at residue 5 of GLIC. In preparation for the Modeler module we aligned the GlyR sequence with the template using the 'align multiple sequences' module of DS 2.1. We used a three-step procedure. First, we used the alignment of GlyR with torpedo nAChR-α1 suggested by Brejc et al. (2001). Second, we used the alignment of nAChR-α1 with GLIC suggested by Bouquet et al. (2009). Third, the resulting alignment of GlyR with GLIC presented a decision point; in the region of interest of the GlyR

sequence there are four gaps in the alignment. This is because GlyR has two more residues in this region than nAChR-α1, which in turn has two more residues than GLIC. To resolve this issue we built three models; the first with the gaps placed by the translation exactly as described above, a second with the four gaps clustered together and placed at the beginning of the beta5-beta5' strand in GLIC, and a third with the four gaps at the end of the beta5-beta5' strand. The three resulting models had all the new loops optimized and then all side chain rotamers automatically optimized. A restraining harmonic potential of 10 kcal/Å^2 was applied to all backbone atoms for the following steps. The models were optimized to a gradient of 0.001 kcal/ Å with a 20 Å nonbond cutoff and then relaxed with molecular dynamics at 300 K for 5,000,000, 2 fs steps using the CHARMm force field.

2.7 Reagents

All reagents used were obtained from Sigma Aldrich (St. Louis, MO). *Xenopus laevis* were purchased from Xenopus Express (Homosassa, FL) or Nasco (Fort Atkinson, WI). Glycine, taurine, strychnine and picrotoxin solutions were made by dissolving the necessary amount into sterile 1x MBS. When not in use stock solutions were kept at 4^o C and used within a week. To decrease degradation, DTT, HgCl2, H_2O_2 and I_2 were made just before application.

2.8 Statistical analysis

Statistical analyses were performed using SigmaStat (Chicago, IL). Concentration-response curves were fit to a four-parameter logistic equation in SigmaPlot 11 to determine the Hill coefficient and EC_{50} values. One-way ANOVAs were used to test for statistically significant changes ($p < 0.05$) in GlyR responses after application of oxidizing, reducing or cross-linking agents.

CHAPTER 3: DISRUPTION OF AN INTERSUBUNIT ELECTROSTATIC BOND IS A CRITICAL STEP IN GLYCINE RECEPTOR ACTIVATION[1]

3.1 Introduction

Glycine receptors (GlyR) are anion-conducting members of the cys-loop receptor superfamily. All members of this cys-loop family are pentameric in structure, with their subunits arranged around a central ion pore. Each subunit consists of a large N-terminal ligand binding domain and four transmembrane segments (TM1-TM4); TM2 of each subunit lines the central ion pore (Langosch et al., 1988). When glycine binds to initiate channel opening it does so by interacting with specific amino acids located at the interfaces between subunits. Spontaneous openings of the integral ion channel of the GlyR do not occur in the absence of neurotransmitter (Twyman et al., 1991). Six loops of amino acids located on adjacent subunits constitute the known binding site for glycine. On the plus (+) side of the interface on one subunit are found loops A-C, whereas loops D-F are located on the minus (–) side of an adjacent subunit (Lynch JW, 2004). In the related nAChR, signal transduction after agonist binding has been described as a

[1] Significant portion of this chapter has previously been published in the journal Proceedings of the National Academy of Sciences (PNAS) in an article titled "Disruption of an intersubunit electrostatic bond is a critical step in Glycine Receptor activation." *Jelena Todorovic, Brian T. Welsh, James R. Trudell, Edward Bertacinni and S.John Mihic © Proc. Natl. Acad. Sci. USA* 107:7987-7992, 2010

"Brownian conformational wave" that travels down the interface between the subunits (Purohit et al., 2007). This study used ϕ analyses to show that the binding pocket region of the *N*-terminal domain is the first to move after ligand binds. Loops 2 and 7 (the cys-loop) of the N-terminal domain interact with the extracellular end of TM1 and the TM2-3 linker region to transmit binding signals to the channel gate (Unwin N, 2005, Bocquet et al., 2009, Lee et al., 2005). In the α1 GlyR subunit, D148 in loop 7 forms an electrostatic bridge with K276 in the TM2-3 linker (Schofield et al., 2003). An interaction of extracellular domain loops with the TM2-3 linker has also been identified in the nAChR (Lee et. al, 2005).

Our previous work demonstrated that mutation of D97 in loop A results in spontaneous channel opening (Beckstead et al., 2002). This D97 residue is conserved in all members of the cys-loop receptor superfamily (**Figure 3.1**), suggesting its critical role in channel function. Because this is a charged residue we asked if it was forming an electrostatic bridge to help maintain the channel in a stable closed state in the absence of neurotransmitter. In this paper we report an interaction between specific charged amino acid residues at the interfaces of adjacent subunits that contribute to the stabilization of the closed channel state. Disruption of these electrostatic bonds is a critical step in GlyR activation.

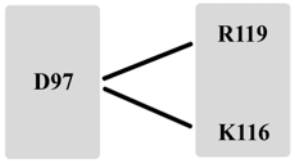
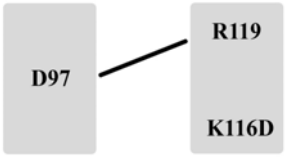
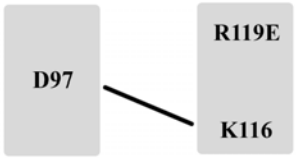
α1 GlyR Mutation	Response to Glycine (EC_{50})	Hill Coefficient	Possible Electrostatic Bonds
None (wildtype)	0.196 mM (n=6)	1.4	
D97R	1.9 mM	0.41	
K116D	3.0 mM (n=6)	1.01	
R119E	87.9 mM (n=6)	1.30	
D97R/K116D	1.3 mM (n=10)	0.64	
D97R/R119E	401 mM (n=6)	3.53	

Table 1. **Characterization of glycine concentration-response relationships of wildtype and mutated α1 GlyR expressed in Xenopus oocytes.** For each receptor glycine concentration-response curves were generated and the glycine EC50s and Hill coefficients determined. The possible inter-subunit electrostatic interactions among the amino acids studied (97, 116 and 119) are shown for each GlyR.

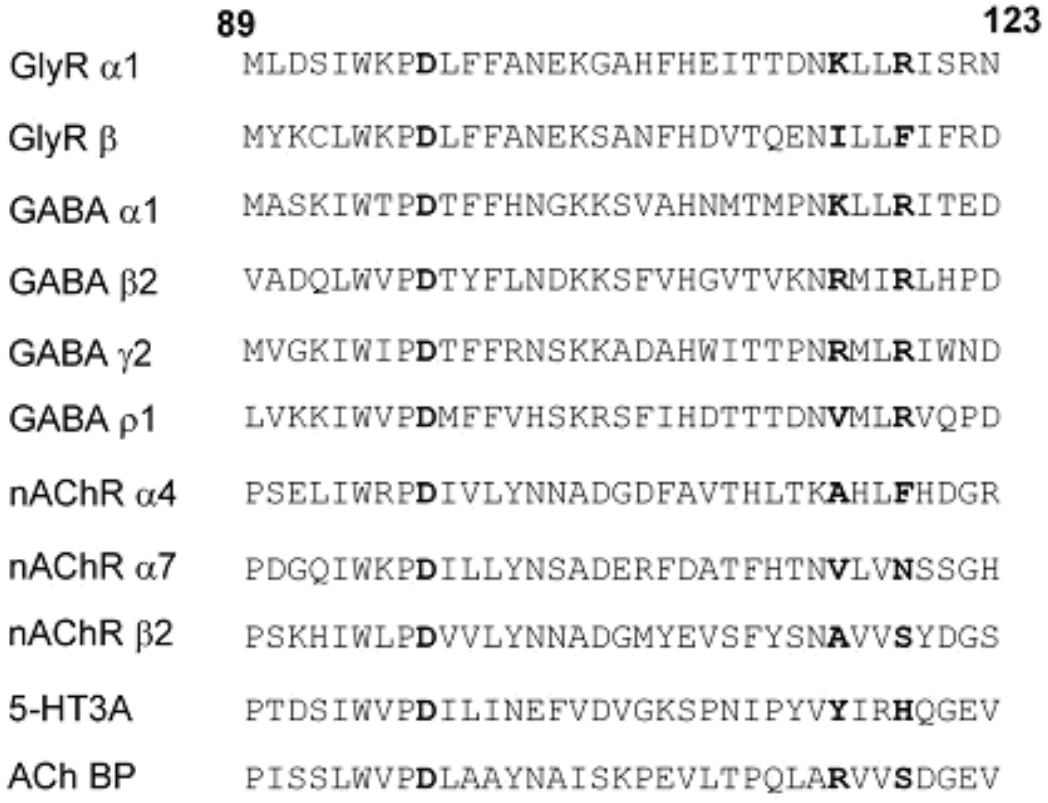


Figure 3.1 Partial amino acid sequence alignment of selected members of the nAChR receptor subunit superfamily. The human GlyR α1 subunit sequence from residues 89 to 123 was compared with the same regions of selected human $GABA_A$, nACh and 5-HT3 receptor subunits, as well as the *Lymnaea Stagnalis* acetylcholine binding protein. Residues equivalent to GlyR α1 D97, K116 and R119 are highlighted.

3.2 Methods

Primer design, Xenopus leavis oocyte isolation and injection, whole cell electrophysiology, single channel data acquisition and analysis have all been previously described (see chapter 2: Experimental Procedures). Details of the reduction and crosslinking experimental procedures are described below.

In general, glycine maximal currents were recorded at least three times to ensure a stable response. The reducing agent 1,4 dithiothreitol (DTT) at a concentration of 10 mM was then applied for 3 minutes. After a 10-12 minute washout, a maximally effective glycine concentration was applied again at least twice before we proceeded with crosslinking or oxidation reactions, depending on the protocol. Next a 10μM concentration of a crosslinking agent mercury (II) chloride ($HgCl_2$) was applied for 1 minute after a stable glycine current has been established just like explained above. After a 10-12 minute washout the maximal glycine current was measured again. In the same way, to measure the disulfide bond formation when in an oxidizing environment, 10 μM iodine (I_2) or 0.3% hydrogen peroxide (H_2O_2) were applied for 1 minute. As noted before, after a washout period of 10-12 minutes, the maximal glycine response was recorded again in order to see whether, and to what extent, receptor activity was altered.

3.3 Results

3.3.1 Charge swap mutations illustrate interactions between D97 and K116/R119

In homomeric α1 GlyR mutating aspartate-97 to arginine (D97R) produces receptors that exhibit increased background inward currents in the absence of glycine, characteristic of spontaneously active channels. This tonic current, not seen in wildtype α1 GlyR, can be blocked by the competitive antagonist strychnine (**Figure 3.2 and 3.3**). Two other mutations, α1 K116D and α1 R119E, each resulted in receptors with decreased glycine sensitivity (**Table 1**), but did not exhibit spontaneously opening channels. The charge reversal mutant α1 D97R/K116D bearing both mutations on the same subunit showed a partial α1 D97R phenotype, with reduced holding currents and a smaller effect of strychnine in inhibiting that tonic current, compared to α1 D97R (**Figure 3.4 a and 3.5**). In contrast, the α1 D97R/R119E charge reversal appeared to stabilize the receptor in the closed state, characterized by wildtype-like whole-cell holding currents as well as a loss of the channel-closing strychnine effects seen in tonically-activating receptors (**Figure 3.4b and 3.5**). Despite the low sensitivity to glycine seen in the α1 R119E and α1 D97R/R119E mutants, the enhancing effects of a very high concentration of glycine (500 mM) could be antagonized by 1-10 mM strychnine (**Figure 3.4 b**). This high concentration of glycine acted specifically via the GlyR on these mutants since concentrations as high as 1 M applied to uninjected oocytes did not elicit any currents. Direct strychnine effects were seen in the α1D97R and α1D97R/K116D mutants, but not in the α1 wildtype, α1K116D, α1R119E or

α1D97R/R119E mutants. Consistent with these findings, the average holding currents when cells were clamped at –70 mV were much higher in oocytes expressing the α1D97R or α1D97R/K116D receptors, reflective of considerable spontaneous channel-opening activity (**Figure 3.5**).

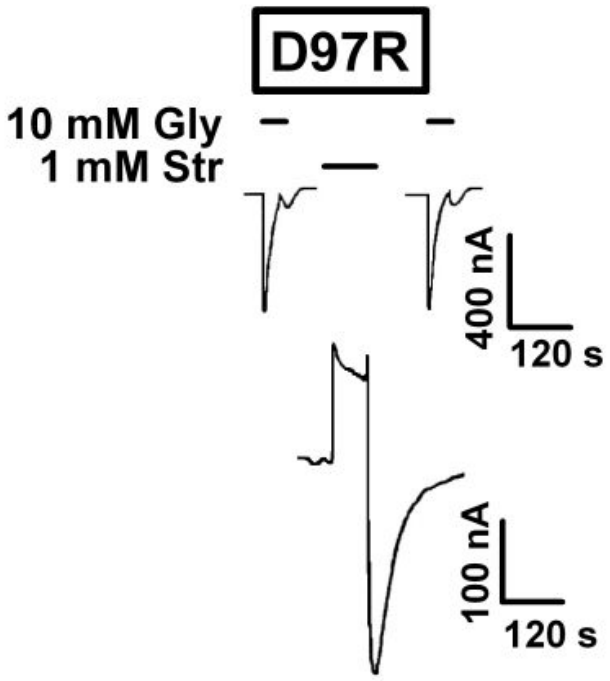


Figure 3. 2: Constitutive activity observed in D97 GlyRα1 mutants. Representative tracings showing the responses to maximally effective glycine and strychnine concentrations for the D97R α1 GlyR mutant. The lines over tracings refer to drug applications. Glycine applied for 45 sec produced inward currents indicating channel opening in the D97R mutant. In contrast, strychnine applied for 1 min initially produced an outward current indicative of closure of spontaneously active channels, followed by an inward current once strychnine was removed

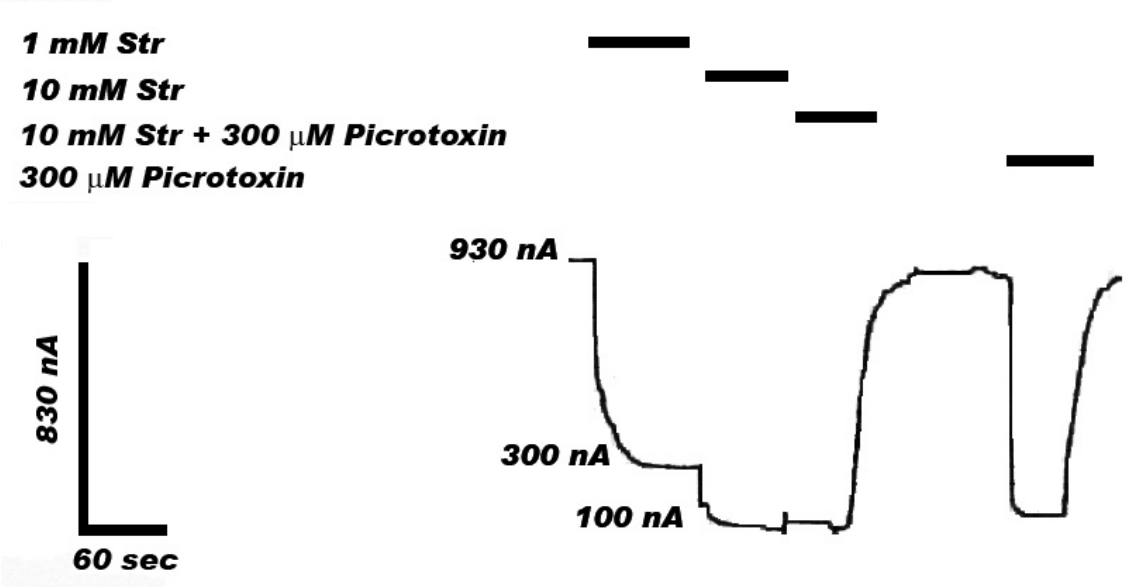


Figure 3.3 α1D97R spontaneous leak currents can be completely abolished by antagonists As depicted in the figure above, this representative tracing shows that with increasing strychnine concentrations (1 to 10 mM) most of the α1 D97R spontaneous current can be prevented. Similar results are seen when picrotoxin (300 μM), another antagonist of the GlyR, is applied.

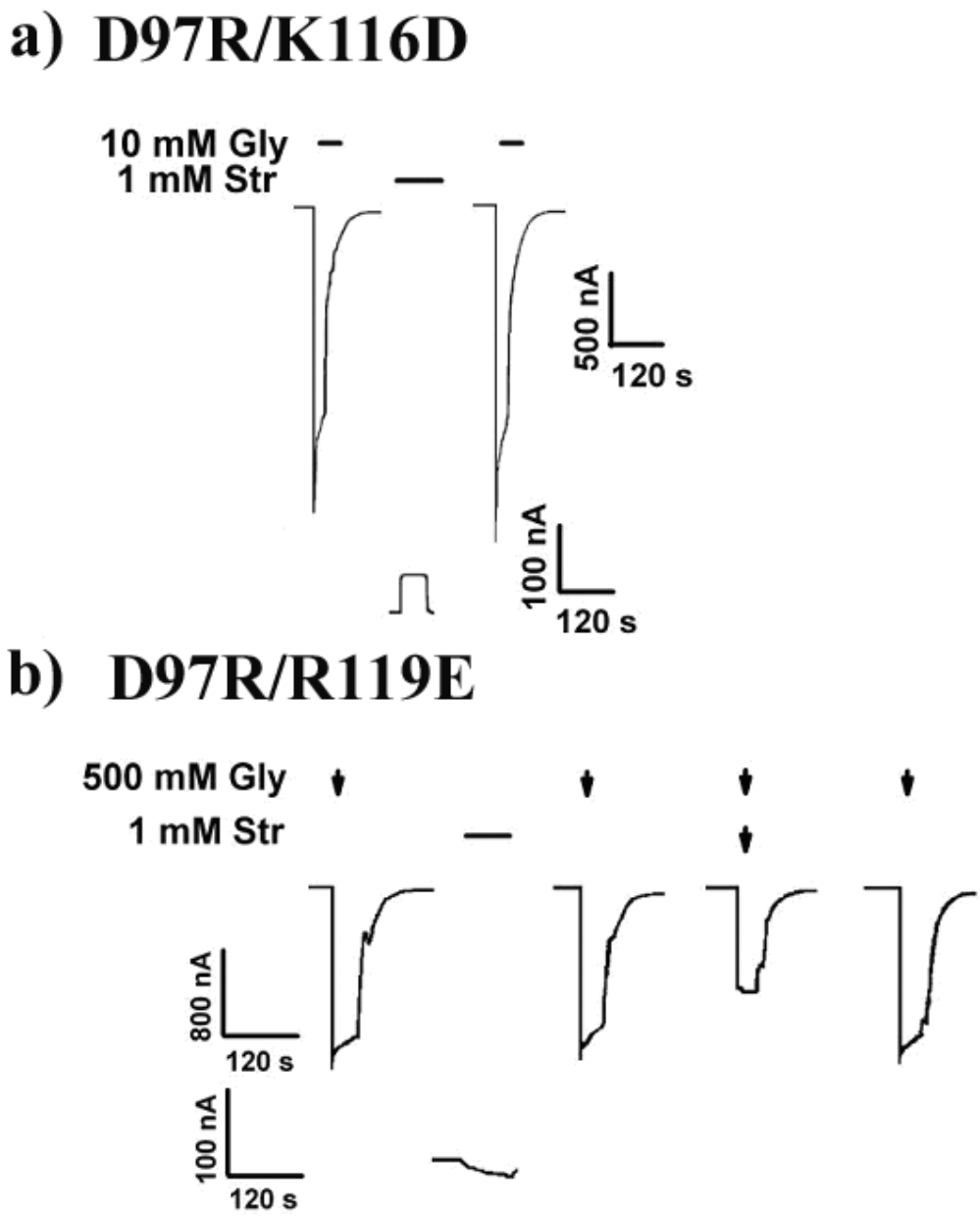


Figure 3.4 Representative tracings showing the reduction of spontaneous opening on D97R/K116D α1 GlyR and a lack of tonic activity on the D97R/R119E double mutant. a) The double mutant D97R/K116D also displays spontaneous channel-opening activity that can be antagonized by strychnine. **b)** The D97R/R119E double mutant shows no evidence of strychnine antagonism of spontaneous activity in whole-cell recordings, although strychnine can block the effects of glycine on these receptors.

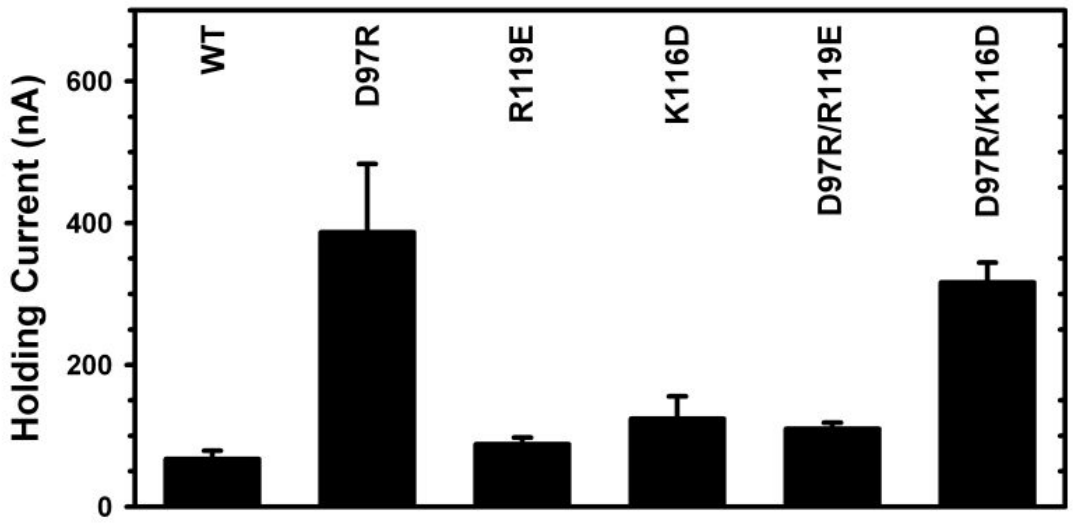


Figure 3.5 Bar graph representation of differences in spontaneous current levels in wt, D97R, R119E, K116D and the double mutants D97R/R119E, D97R/K116D. The holding current required to voltage clamp oocytes at –70 mV was measured and compared among mutants. Wildtype α1 GlyR do not exhibit spontaneous channel opening and typically exhibit holding currents less than 100 nA. These low holding currents were also seen in the R119E, K116D and D97R/R119E mutants. However, the D97R and D97R/K116D mutants displayed significantly greater holding currents. Values are reported as mean ± S.E.M. of 8-17 oocytes.

3.3.2 Single channel recordings of D97R and D97R/R119E mutants

In research conducted by Brian T. Welsh he used outside-out patch clamp recordings of homomeric α1 D97R GlyR and α1 D97R/R119E GlyR in the absence of exogenously applied glycine, as well as wildtype α1 GlyR exposed to 10 mM glycine (**Figure 3.6**), in order to further study these mutants and the interactions of D97 and R119. The spontaneous activity observed in the D97R GlyR appeared quite similar to the effects of a saturating concentration of glycine on wildtype receptors. The D97R/R119E receptor behaved very differently, with many very brief opening events being seen as shown in the tracing on the left-hand side of **Figure 3.6 c**. The two mutant receptors displayed quite different open and closed dwell-time properties. D97R receptor spontaneous activity had a P_{open} of 0.91, very similar to that produced by glycine on wildtype GlyR (P_{open} = 0.9). In contrast, the D97R/R119E mutant had a much lower P_{open} of 0.14, which is likely an over-estimate since multiple channels probably contributed to this measure. Open dwell-time histograms generated from the tracings were fit using two open-time components (τs) for the wildtype, α1 D97R GlyR and α1 D97R/R119E GlyR **(Figure 3.6, right panels)**. Spontaneous openings were much longer in the D97R mutant, suggesting that re-establishing the electrostatic bond in the double D97R/R119E mutant acts to stabilize the closed state of the channel, albeit imperfectly. Supporting this argument, the histograms illustrating the fits of the closed-dwell times for α1 D97R GlyR and α1 D97R/R119E GlyR, which were each fit using two closed-time components, indicate that longer closed times were much more evident in the double mutant, while very short closings predominated in the D97R mutant.

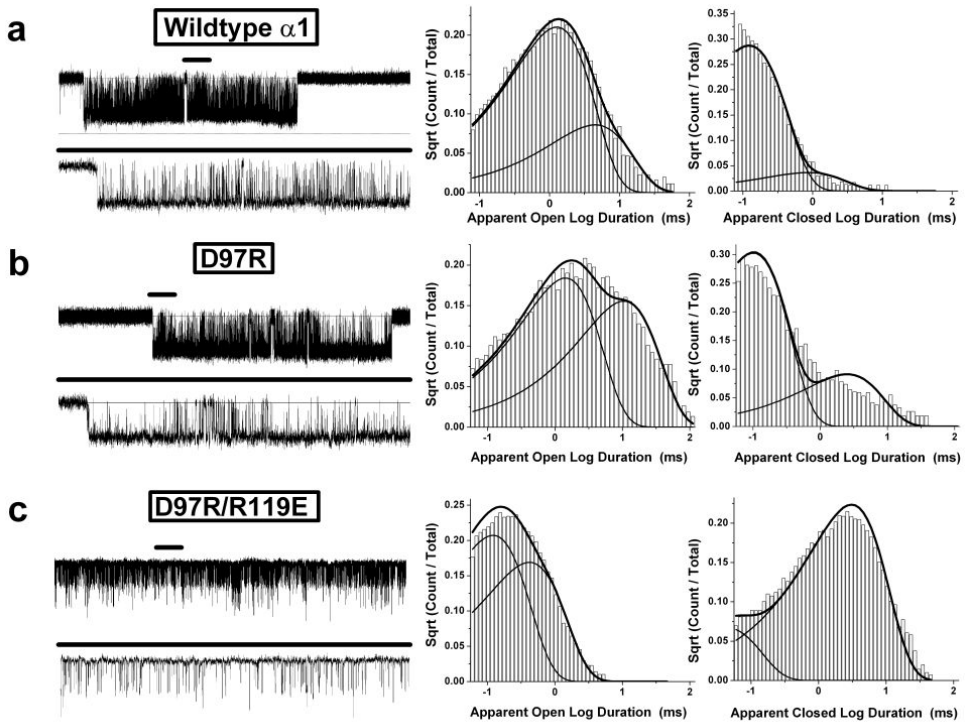


Figure 3.6 Single-channel recordings of D97R and D97R/R119E α1 GlyR mutants. Representative tracings of single channel recordings made from **a)** wildtype α1 GlyR exposed to 10 mM glycine, as well as spontaneous activity recorded from **b)** D97R α1 GlyR and **c)** D97R/R119E α1 GlyR in the absence of exogenously-applied glycine. Each of the top lines represents 7 sec of recording and regions with the horizontal lines above them in each trace are expanded below. Downward deflections signify channel opening. The D97R/R119E trace appears to show multiple channels in the patch with very brief spontaneous openings. Open- and closed-time histograms generated from measurements of the wildtype GlyR in the presence of 10 mM glycine appeared quite similar to those describing the spontaneous activity of the D97R α1 GlyR. Both exhibited longer open times as well as shorter closed times than the D97R/R119E double mutant. Activity of each GlyR was adequately fit using two open-time or closed-time components (τs). The two thin lines in each histogram describe the individual components while the thicker line is an overall fit.

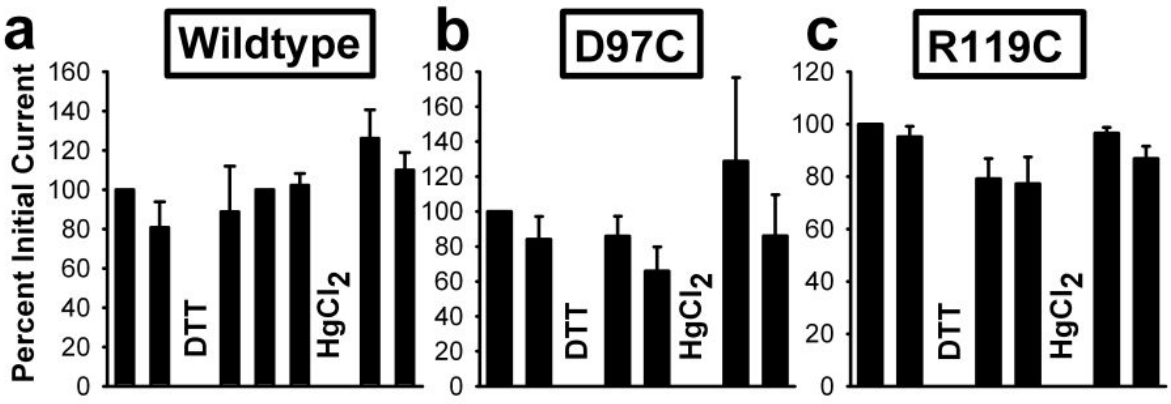


Figure 3.7 Reduction and crosslinking reactions show no significant effects on wt GlyRs or D97C and R119C single mutants. Control bar graphs showing that no significant changes in current are recorded on the **a)** wt, **b)** D97C and **c)** R119C GlyRs after application of either a reducing agent DTT or a crosslinking agent $HgCl_2$

3.3.3 Effects of reduction and cross-linking on cysteine mutants

To test our hypothesis that residues D97 and R119 are physically interacting, we constructed α1 D97C, R119C and D97C/R119C mutants to test for possible cross-linking. In all cross-linking experiments, responses to maximally effective concentrations of glycine (1 M) were first measured twice. After establishing stable glycine responses, the reducing agent DTT, the cysteine-bridging agent $HgCl_2$ or the oxidizing agent iodine were tested for their effects. Applications of 1 M glycine were 3-5 sec long, during which a peak response was observed, followed by 12 - 15 min washout periods. Oocytes were then perfused with 10 mM DTT for 2 minutes and after a 12 min washout 1M glycine was reapplied twice. Oocytes then received either a 2 minute application of $HgCl_2$ (10 μM), or a 1 minute application of iodine (0.5 mM), which favors disulfide bond formation. Control experiments followed the same experimental procedure but with the application of buffer (MBS) instead of DTT, $HgCl_2$, or iodine. The wildtype, D97C and R119C single mutants showed no significant effects of either DTT or $HgCl_2$ **(Figs 3.7 a, b and c)**. In marked contrast, application of DTT to the α1 D97C/R119C double mutant significantly enhanced responses to glycine applied afterwards, while $HgCl_2$ decreased the magnitude of glycine effects **(Figs. 3.8 a and 3.8 b)**. Re-application of DTT after $HgCl_2$, resulted in the same high glycine-mediated currents that were observed after the initial DTT application.

All three mutants were also tested for possible disulfide bond formation by the oxidizing agents iodine. In both the D97C and R119C mutants, the application of 0.5 mM iodine for 1 min had no effects on glycinergic currents, suggesting that there were

no other available free cysteine residues close enough to form disulfide bonds. However, the double mutant D97C/R119C was able to form a disulfide bond when in an oxidizing environment since iodine (**Figure 3.8 c**) markedly decreased the effects of glycine, in a DTT-reversible manner. In order for this disulfide bond formation to occur the sulfhydryl groups of cysteine residues can be up to 15 Å (Careaga et al., 1992) apart; once the bond has formed the distance between α-carbons in the two cysteines is 5.6 Å (Schmidt et al., 2007).

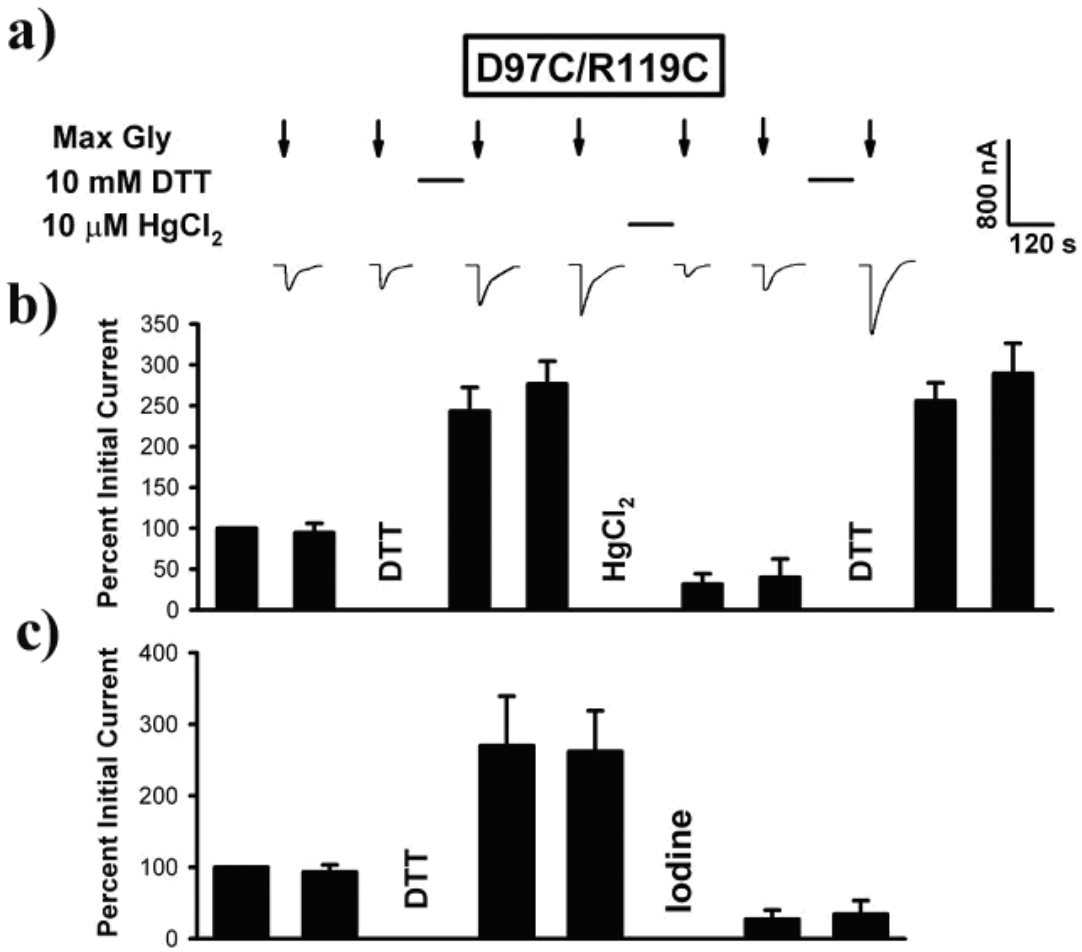


Figure 3.8 Reduction, crosslinking and oxidation of the double mutant D97C/R119C In contrast to the wt and single mutants data, **a)** the sample tracing of the double mutant D97C/R119C α1 GlyR shows a significant increase in glycine current after exposure to DTT and a decrease in the glycinergic current after application of the cross-linking reagent $HgCl_2$. **b)** Bar graph illustrating the effects of DTT and $HgCl_2$ on D97C/R119C α1 GlyR responses. Data are reported as the mean ± S.E.M. of 5-12 oocytes. **c)** The oxidizing agent iodine can also decrease the effects of a maximally effective glycine concentration. Data are reported as mean ± S.E.M. of 6 oocytes.

3.3.4 Inter-subunit interactions stabilize the closed state of the receptor

We next sought to determine whether the interaction between D97 and R119 occurs between adjacent subunits and not within the same subunit. To determine this we mixed cDNAs containing two different single mutants, one expressing the α1 D97C mutation alone and the other expressing the α1 R119C mutation **(Fig. 3.9).** The D97C and R119C cDNAs were mixed in a ratio of 1:50, allowing us to predict the percentages of D97C and R119C subunits in different receptor populations that would be expressed on the oocyte membrane surface. The equation

$$P = 100 \text{ X } p_{D97C}{}^{n} \text{ X } p_{R119C}{}^{(5-n)} \text{ X } 5! / [n!(5-n)!]$$

describes the percentage of receptors[12] bearing n D97C subunits, where n = 1 to 5. p_{D97C} and p_{R119C} represent the fractions of D97C and R119C cDNAs injected into oocytes; those numbers were 1/51 and 50/51, respectively. Thus the chances of obtaining receptors bearing zero, one or two D97C subunits in any individual receptor were 90.6%, 9.06% and 0.34%, respectively. Our studies of the homomeric α1 R119C single mutant showed that it is markedly insensitive to glycine, such that concentrations of glycine below 1 mM elicit no currents. In contrast, co-expression of the D97C and R119C mutants resulted in receptors displaying considerable currents at glycine concentrations as low as 10 μM (Fig. 3.10b). It seems that replacement of C119 with the wildtype R119 in even one of five subunits in a receptor is sufficient to restore sensitivity to low concentrations of glycine. Co-expression of α1 wildtype + R119C cDNAs at a 1:50

ratio produced the same results (Fig. 3.10b). This suggested that the responses obtained using low concentrations of glycine in the α1 D97C + R119C heteromeric GlyR must have been elicited by receptors that bore one copy of the D97C mutation and this receptor is illustrated in **Figure 3.9**. It possesses a single wildtype D97-R119 inter-subunit bond, a single D97C-R119C bond as well as three D97-R119C bonds. A concentration of 30 μM glycine was applied to D97C + R119C receptors to ascertain the effects of $HgCl_2$ and DTT on cross-linking between subunits each bearing either D97C or R119C mutations (**Figure 3.10a, c**). Initially these receptors displayed low μA currents in response to 30 μM glycine, which decreased significantly after $HgCl_2$ exposure, suggesting cross-linking between adjacent subunits bearing α1D97C and α1R119C mutations. This cross-linking was reversed by DTT.

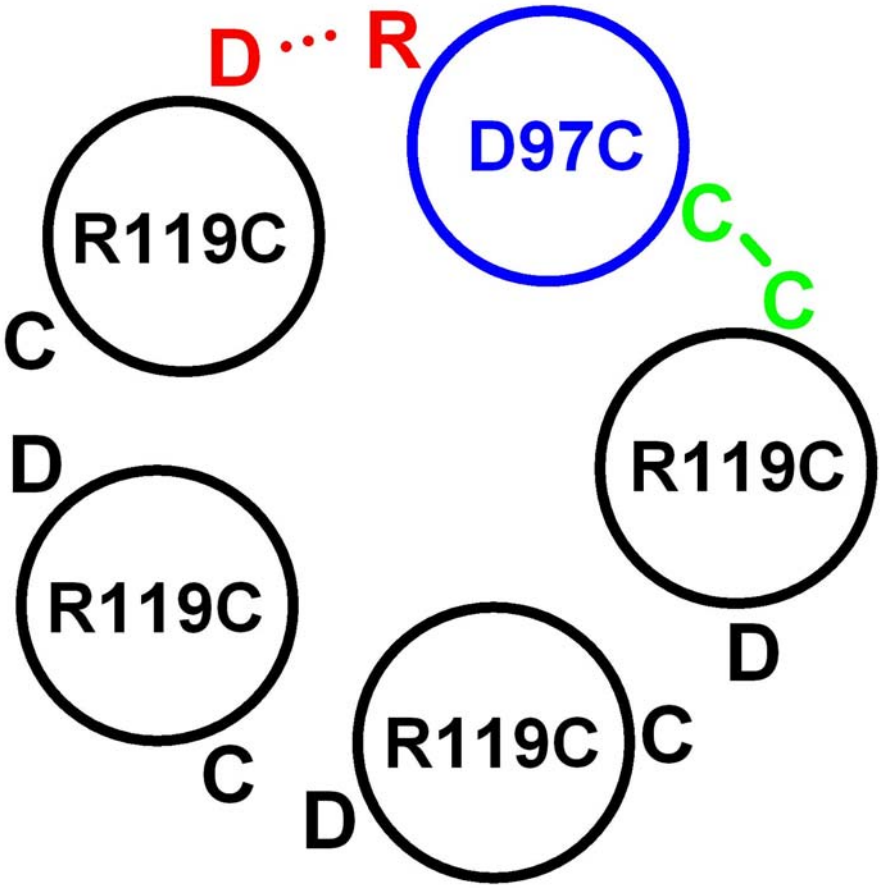


Figure 3.9 Graphical representation of the α1 GlyR expressing one D97C and four R119C mutant subunits In this heteromeric receptor only one disulfide bond could form (between D97C and R119C subunits: color coded green) while one interface would exhibit wild type binding pocket (between D97C and R119C: color coded red).

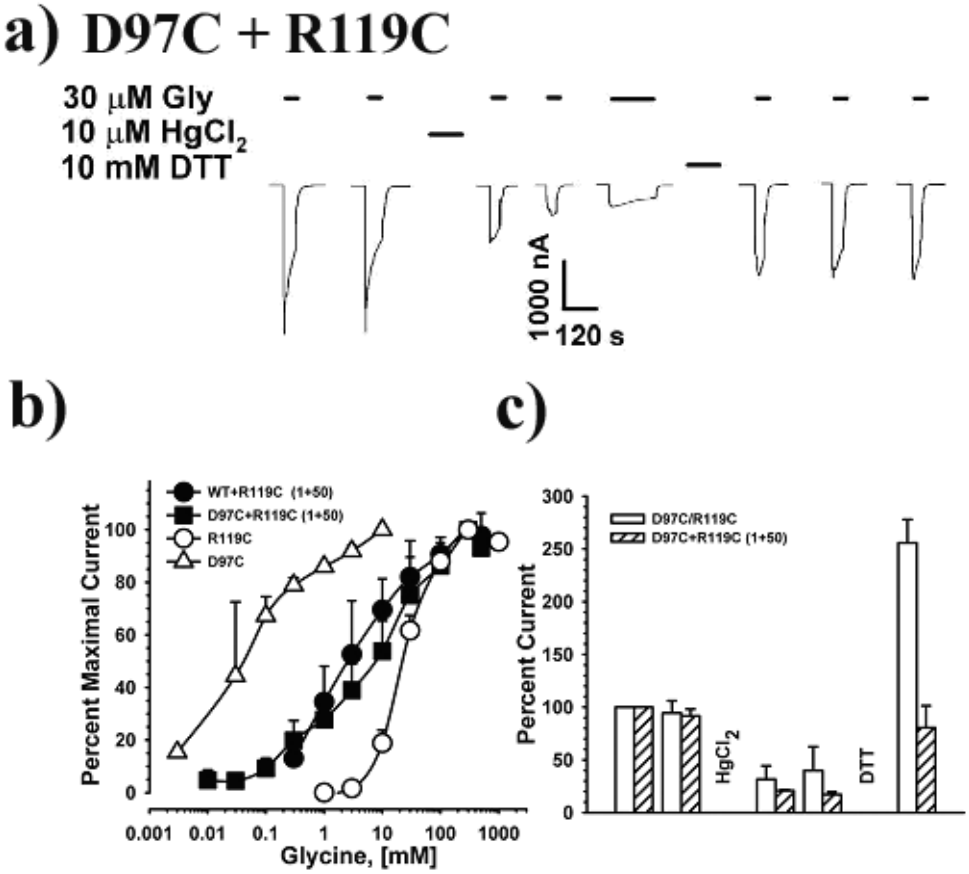


Figure 3.10 Disulfide bond formation between adjacent subunits. a) A concentration of 30 μM glycine does not activate homomeric R119C α1 GlyR. However, in the D97C+R119C (1:50) receptors, in which the two single mutants D97C and R119C were injected in a cDNA ratio of 1:50, 30 μM glycine elicited currents. These currents are believed to be produced by receptors bearing four R119C subunits and a single D97C subunit. **b)** Glycine concentration response curves were constructed for wildtype+R119C (1:50) and D97C+R119C (1+50) receptors, in which the R119C subunit cDNA was co-injected with either the wildtype or D97C cDNAs but at 50 X the concentration. The glycine concentration-response curves thus depict the responses of homomeric R119C receptors, which are very insensitive to glycine, as well as a minority of heteromeric wildtype+R119C or D97C+R119C GlyR that exhibit greater sensitivity to glycine. The 10,000-fold glycine concentration range at which glycine effects are observed is evidence of this receptor heterogeneity. For wildtype+R119C GlyR the EC_{50} was 3 mM, with a Hill coefficient of 0.69, while for the D97C+R119C GlyR the EC_{50} and Hill numbers were 8.6 mM and 0.55, respectively. **c)** Bar graph summarizing data comparing the effects of $HgCl_2$ and DTT on homomeric receptors bearing both the D97C and R119C mutations on each subunit (D97C/R119C) as well as those bearing the two mutations on different subunits, at D97C+R119C ratios of 1 to 50. Data are shown as mean ± SEM obtained from 3 oocytes.

3.3.5 Co-expressing α1D97C/R119C with the wt β GlyR subunit can prevent crosslinking between the subunits

When we co-expressed α1 GlyR subunit carrying a double mutant D97C/R119C mutation with the β GlyR subunit, in the 1:10 ratio we saw increased holding current when compared to either wt or α1D97C/R119C mutant receptors **(Figure 3.11).** The spontaneous leak of current was evident and measurable as an outward current observed after application of the GlyR antagonist PTX at a concentration of 300 μM for 1 minute. We first demonstrated stable glycinergic currents by measuring the maximally attainable currents two or three times before continuing with the experiment. The washouts between glycine applications lasted 15 minutes due to the high concentration of the agonist used. When the reducing agent DTT was applied to the oocytes expressing this mixture of subunits (10 mM for 3 minutes) there was no change in the amount of current attainable by the following 1M glycine application. The same was true with the crosslinking reaction. When 10 μM $HgCl_2$ was applied for 1 minute no significant change in current was detected after the oocytes were exposed to the crosslinking agent **(Figure 3.11).**

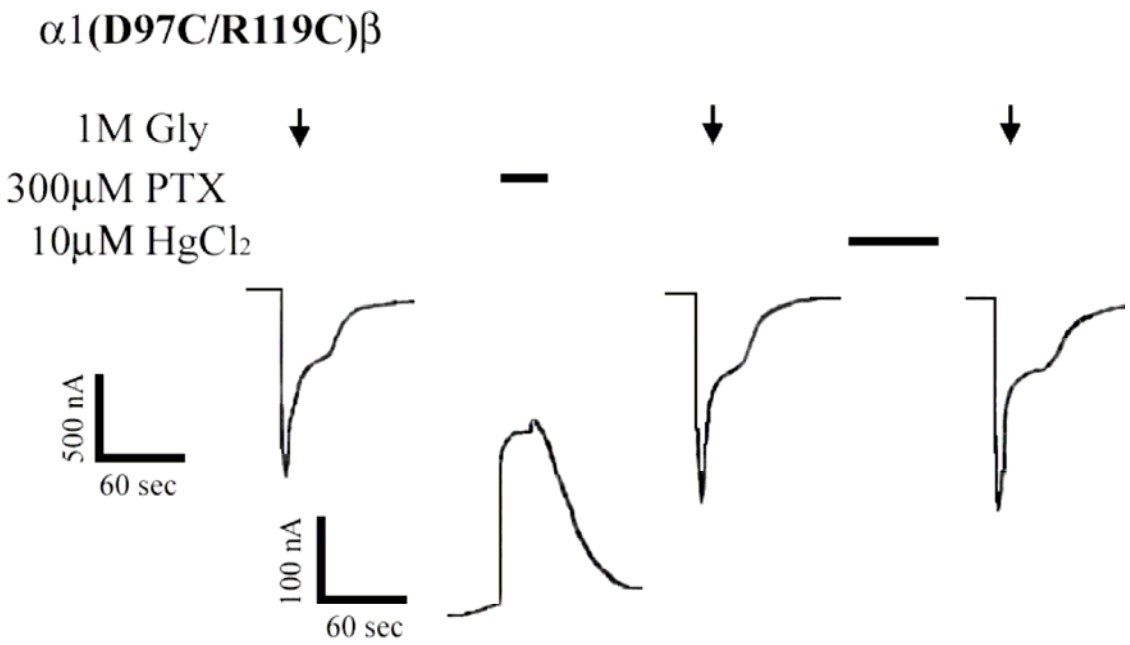


Figure 3.11 Sample tracing demonstrating the loss of the D97-R119 interaction in α1(D97C/R119C)β GlyRs Although we can see that the presence of the α1R119 mutation still forms receptors that require 1M glycine to elicit currents, traces show that receptors are still spontaneously open: the bottom trace shows the effects of 300 μM PTX on these receptors. The crosslinking agent $HgCl_2$ does not have an effect on these heteromeric receptors most likely due to a physical separation of α1(D97C/R119C) subunits with intercalating β wt subunits.

3.3.6 Modeling

Initial use of the homology model, conducted by James Trudell and Edward Bertacinni, was to predict residues in the vicinity of D97 that could form a salt bridge with it. They considered residues that would be suitable for charge-reversal mutations. A second use, as described in Methods, was to refine the alignment of the GlyR sequence with that of the template, GLIC **(Figure 3.12a)**. The ambiguity in alignment is that GlyR has four more residues in the region of interest than does GLIC. Although many other regions in the total alignment have important conserved residues or prominent secondary structural features (Bertaccini and Trudell, 2002), the region of interest here does not. Even secondary structure prediction for GlyR in this region does not help because it is an interrupted β strand, designated β5- β5' (throughout we use the nomenclature suggest for the nicotinic acetylcholine binding protein by Brejc et al. (2001). We built models based on three alignments; the four gaps spaced along the β5- β5' strand of GLIC, the four gaps clustered after β5' (increased the size of Loop 6), and the four gaps clustered before β5 (increased the size of Loop 5). Only the latter alignment produced a good model because Loop 5 could expand without bad overlaps with other residues **(Figure 3.12b)**. In contrast, the former two models resulted in conflicts with other regions; in particular the expanded Loop 6 intercalated with Loop 3.

We then used the homology model to observe possible interactions of D97 with K116 and R119 on the opposite subunit interface. **Figure 3.12b** shows that both K116 and R119 are in proximity to D97 and that fluctuations of torsion angles within each side chain produce a variety of possible electrostatic interactions. In fact, we observed

variations in conformations in the other four subunit interfaces that are not shown in **Figure 3.12b**. It is interesting that the model started out as a homopentamer, but after 5,000,000 steps of molecular dynamics it diverged such that the relative positions of D97, K116, and R119 were slightly different at each interface.

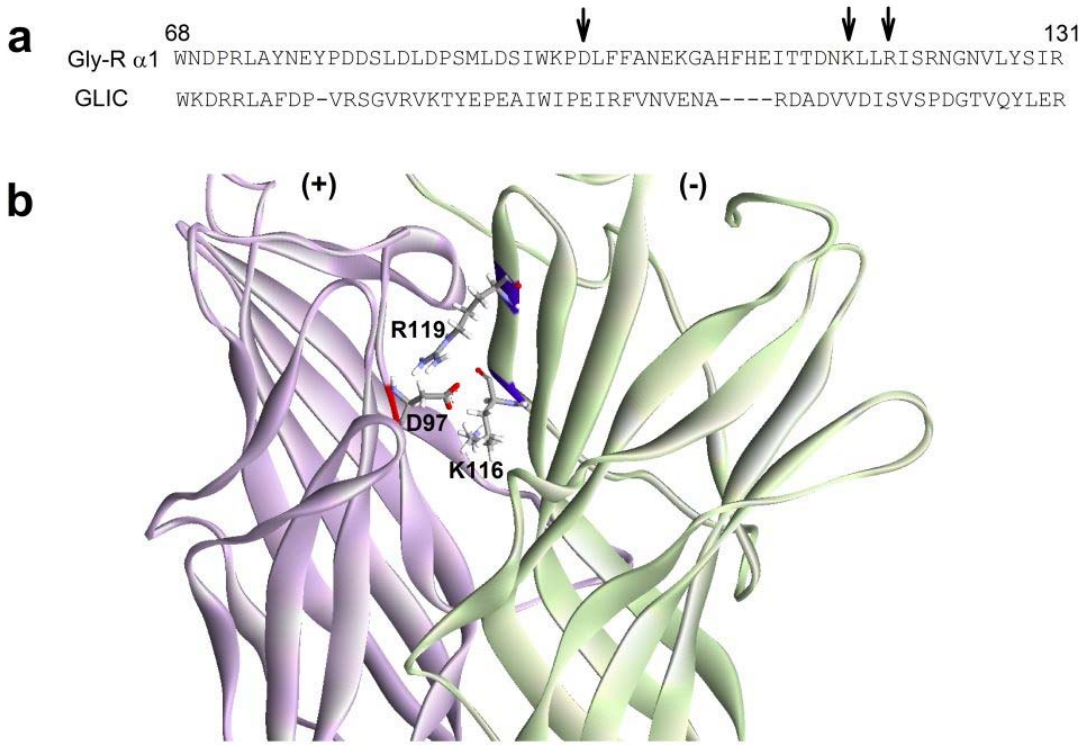


Figure 3.12 Homology model of the GlyR inter-subunit interface.
a) A partial amino acid sequence alignment of the GlyR α1 (residues 68 through 131) and GLIC sequences is provided. Arrows highlight D97, K116 and R119 in the GlyR α1 sequence. **b)** A homology model of GlyR was built as described in Methods.

3.4 Discussion

It is critical for the proper regulation of neurotransmission that ligand-activated ion channels remain closed until a binding signal is received by the receptor. The receptor complex must exist in conformations that will keep the channel from opening in the absence of agonist as well as allow for rapid conformational changes to occur within microseconds after neurotransmitter binding. The opening of the GlyR pore is thought to be initiated by the binding of the glycine molecule at interfaces between loops A, B and C, on the "+" side of one subunit and β sheet segments D, E and F on the "-" side of an adjacent subunit (Lynch JW, 2004). The binding of glycine is stabilized by its interactions with the side chains of a number of amino acids in the "+" and "-" sides of adjacent subunits, including R119 (Lynch, 2004, Rajendra et al., 1995, Grudzinska et al., 2005, Grudzinska et al., 2008). The 2BG9 model of the nicotinic acetylcholine receptor (Unwin, 2005) and the prokaryotic acetylcholine receptor homolog GLIC (Bocquet et al., 2009) provide a structural framework to aid in explaining how ligand binding signals could be transmitted to the pore (Unwin, 2005). From binding to opening, electrostatic interactions are thought to occur between residues in extracellular loops 2, 7 (the conserved cys-loop) and 9, with amino acids in the pre-TM1 region, the TM2-3 extracellular loop, and post-TM4 residues (Xiu et al., 2005), linking ligand binding to channel opening. In the $GABA_A$ receptor specific electrostatic interactions between D57 and D149 residues in loops 2 and 7 with K276 in the TM2-3 linker region affect gating (Kash et al., 2003); later work also implicated a residue in the pre-TM1 region (Kash et

al., 2004). However, in the homomeric α1 GlyR, direct electrostatic interactions between D53 or E57 of loop 2, or D148 of loop 7, with K276 in the TM2-3 linker were not observed (Absalom et al., 2003). Xiu et al. (2005) concluded that interactions between extracellular domain amino acids with those in pre-TM1, TM2-3 and post-TM4 do not generally appear to involve specific amino acids but rather that overall clusters of positively and negatively charged residues mediate interactions between these domains. In a series of publications Auerbach and colleagues studied the relative timing of movements of domains of the nAChR initiated by ligand binding that result in the transitioning of channels from closed to open states. Upon acetylcholine binding the transmitter binding region involving loops A, B and C moves first (Grosman et al.,2000), loops 2 and 7 then move (Chakrapani et al., 2004, Jha et al., 2007), and this is followed by the almost simultaneous movements of the TM2-3 linker region (Jha et al., 2007) and TM2 (Purohit et al., 2007, Mitra et al., 2005). It is believed that a "Brownian conformational wave" involving sequential movements of domains of portions of receptor subunits ultimately links ligand binding to channel opening.

In the current study we found a fundamental inter-subunit interaction that plays a significant role in stabilizing the closed state of the channel in the absence of agonist activation signals and plays a role in the transduction of binding signals to the integral ion channel pore. We noted that the WxPD motif in which D refers to D97 in the α1 GlyR is invariantly conserved among all members of the cys loop family, including the snail acetylcholine binding protein. Mutation of this highly conserved D97 residue to arginine destabilizes the receptor closed state resulting in channels that exhibit constitutive

activity. We sought to determine if the D97 residue was stabilizing the closed-channel state of the α1 GlyR by forming an electrostatic bond with a positively charged residue, and identified R119 on an adjacent subunit as a plausible candidate, based on molecular modeling. Reversing the charge at D97 to arginine results in tonic channel opening that can be markedly reduced by a second charge reversal mutation at R119. Interestingly the single R119E mutation does not result in spontaneous channel opening and this may be because the D97 residue can also interact with K116. We hypothesize that the electrostatic bond between D97 and R119 (or K116) breaks at the initiation of the binding signal allowing for the uninterrupted propagation of the conformational wave to occur, ultimately leading to the opening of the channel pore. The interaction between D97 and R119 is also illustrated by mutating both residues to cysteine. Application of DTT to D97C/R119C α1 GlyR results in markedly enhanced glycine-mediated currents, consistent with a breakage of disulfide bonds **(Figures 3.8 and 3.10)**. Application of the oxidizing agent iodine or the cysteine-bridging agent $HgCl_2$ decreases the magnitudes of glycine-mediated currents, suggesting that the re-linking of D97C and R119C constrains the channels from opening. Importantly, none of these effects are seen with either the D97C or R119C single mutants or in wildtype receptors.

We next addressed the issue of whether the D97 and R119 interactions were occurring between or within α1 subunit(s). Our mixed cDNA data, involving the co-expression of two α1 GlyR mutants each bearing either a D97C or R119C mutation, show that reduction and cross-linking effects are conserved despite the fact that no single subunit contains two cysteine mutations. When α1D97C and α1R119C cDNAs are

injected in a 1:50 ratio, we would expect about 9% of the resulting receptors to consist of a single α1 D97C subunit and four α1 R119C subunits **(Figure 3.9)**. In this case these receptors would possess three distinct inter-subunit interfaces: one D97C-R119C interface capable of forming a disulfide bond, a single wildtype D97-R119 interface and three D97-R119C interfaces that would not interact either covalently or electrostatically. Our results show that these α1D97C + α1R119C receptors become dramatically more sensitive to glycine compared to α1R119C homomeric mutants; this suggests that the single wildtype-like D97:R119 interface is able to leftshift the glycine concentration-response curve so that it is closer to wildtype GlyR sensitivity. After application of the bridging agent $HgCl_2$, a single covalent link formed between α1D97C and α1R119C on adjacent subunits is sufficient to limit the abilities of these subunits to move relative to one another and this results in a significant decrease in receptor activation **(Figures 3.10a and c)**. The breakage of this disulfide bond by DTT can reverse this. In addition when the α1(D97C/R119C) double mutant was co-expressed with the wild type β GlyR subunit we saw a loss of the crosslinking effects of $HgCl_2$ that were seen in receptors composed solely of D97C/R119C α subunits. These receptors also showed increased holding currents and channel-closing effects of both strychnine (data not shown) and PTX **(Figure 3.11)**. Presumably this offers further proof that the interaction of D97 and R119 occurs between adjacent subunits.

Our single channel recordings on the charge reversal mutant α1D97R/R119E illustrate that restoration of the electrostatic bond in this double mutant prevents the receptor from opening to the same extent as the D97R mutant in the absence of ligand. In

fact the spontaneously opening α1D97R mutant appears to behave very much like a wildtype GlyR exposed to a saturating concentration of glycine. The α1D97R receptor has a P_{open} of 0.91 and, like the wildtype GlyR, openings are grouped into long clusters containing very brief closing events. In addition, in both GlyR clusters appear to be terminated by entries into longer-lived desensitized states. Histograms generated of the open and closed dwell-times are also very similar between spontaneously opening α1D97R and fully activated wildtype GlyR. Spontaneous channel opening was also reported by Miller et al. (2008) who studied the nearby α1F99A mutant. This mutation produced spontaneously opening channels due to a movement in loop A, and we wonder if this mutation may also produce a weakening of the electrostatic bond between α1D97 and α1R119.

By restoring the attractive force between these residues in the α1D97R/R119E GlyR we were able to increase the closed state stability of the double mutant **(Figure 3.6)**. One logical inference that might be made from our findings is that the brief intra-burst closings seen in maximally-activated wildtype α1 GlyR do not involve the temporary restorations of electrostatic bonds such as the one between the D97 and R119 residues. Very brief closings were also prevalent in the α1D97R mutant presumably because of the inability of the channel to re-establish salt bridges that would stabilize longer-lived closed states. Longer-lived closed states seen in wildtype GlyR at lower glycine concentrations might, however, involve restoration of these electrostatic bonds. Interactions other than D97/R119 within this region do occur, such as the hydrophobic interactions noted by Miller et al. (2008) and we also see this in our partial phenotype

mutant α1D97R/K116D, where the tonic activity is reduced but not decreased to the same extent as in the α1D97R/R119E mutant. We hypothesize that this inter-subunit region of charges may be implicated in agonist binding, where the interaction between charged residues on adjacent subunits regulates closed to open state kinetics after agonist binds within the binding pocket. The dramatic reduction of receptor sensitivity to glycine in all α1R119 mutants lends support to this idea. The competing electrostatic interactions shown in **Figure 3.12b** may help explain why the α1D97R mutation was so deleterious whereas the α1K116D and α1R119E mutations were better tolerated from the view of spontaneous opening. This compensating interaction is similar to what was observed between loops 2, 7, and the TM2-3 linker in the $GABA_AR$ (Kash et al., 2003). In fact, Xiu et al. (2005) suggested that such redundant electrostatic interactions represent a common motif for transduction of gating energy. In conclusion, our study demonstrates the importance of inter-subunit electrostatic coupling for normal receptor activation, and that disruption of this electrostatic bond may represent an initial step in GlyR activation.

CHAPTER 4: AN ELECTROSTATIC INTERACTION OF ARGININE-131 WITH ASPARTIC ACID-97

4.1 Introduction

The most recent computer model of the ECD, describing the electrostatic interaction of the aspartic acid residue at position 97 with arginine at position 119, also implicated other residues as possible options for such interaction at the interfaces of subunits. One of these, R131 was particularly interesting since it has previously been implicated in many other aspects of glycine receptor function (Grudzinska et al., 2005). When studying the improved GlyR model, it is obvious that this charged residue is positioned right above the D97-R119 interaction studied previously **(Chapter 3)** and it may be positioned in a manner that allows the formation of a triad of charges with these two residues. Alternatively it may be the one that actually forms a direct electrostatic interaction with aspartic acid at position 97, and in this way contributes to stabilizing the receptor in its wild type closed state. Previous work showed that this residue plays an important role in stabilizing glycine and strychnine binding (Grudzinska et al., 2005).

These findings fit with the hypothesis that this residue may indeed be at the interface between subunits where it forms part of the binding pocket for both glycine and strychnine. In this chapter I show that mutations at this position destabilize the closed state of the receptor resulting in small but significant tonic activity. Interestingly, when

this residue is mutated to either aspartic acid (R131D) or cysteine (R131C), the resulting receptors display almost normal glycine concentration-response curves, but is characterized by significant changes in the relative efficacies of other agonists. Taurine becomes a full agonist on receptors carrying this mutation while efficacy of another beta amino acid, βAIBA, is increased as well. A competitive antagonist nipecotic acid acts as a weak partial agonist with similar pharmacological effects to those seen in the GlyR α1 D97R mutant.

4.2 Methods

Primer design, oocyte harvesting and injection, general electrophysiology techniques and statistical analysis have all been previously described in Chapter 2.

4.3 Results

4.3.1 Characterization of α1 R131D GlyR mutant

Currents elicited by varying concentrations of glycine and taurine were assayed on homomeric R131D α1 GlyR. Concentration response curves were generated and the EC_{50} was determined for both agonists tested. The α1R131D mutation did not significantly affect glycine potency although this charge reversal did produce measurable spontaneous activity **(Figure 4.1a).** This residue was previously shown to play a role in stabilizing strychnine binding, so in order to demonstrate spontaneous activity we recorded and compared the background current from this mutant to those of the wild type GlyRs **(Figure 4.1b).** We also measured a direct effect of 200mM EtOH on the receptors expressing this mutation **(Fig 4.1c)**. . On the other hand, relative to glycine, taurine efficacy increased significantly, and nipecotic acid, normally an antagonist at the wt GlyR became a weak partial agonist **(Figure 4.2).**

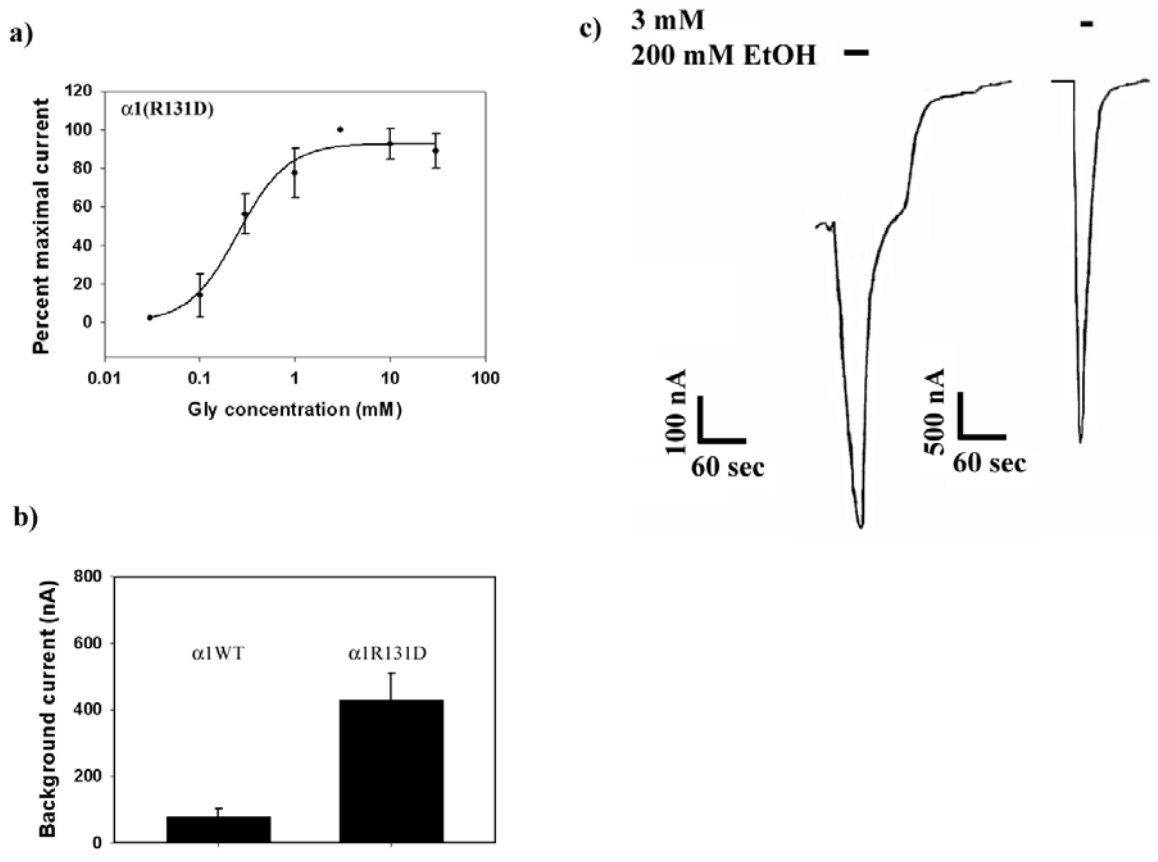


Figure 4.1 Characterization of α1R131D single mutant
a) A glycine **c**oncentration response curve shows that potency of glycine at R131D GlyR is comparable to that of wt GlyRs (EC50 = 271μM and Max = 2.2 mM; n=6) **b)** Holding currents are significantly larger at the R131D mutant than those measured at wt GlyR voltage-clamped at –70 mV **c)** Significant direct effect of 200mM EtOH applied in the absence of glycine on the receptors expressing α1 R131D mutation. This is very similar to what was reported by Beckstead et al. (2002) for the D97R mutation.

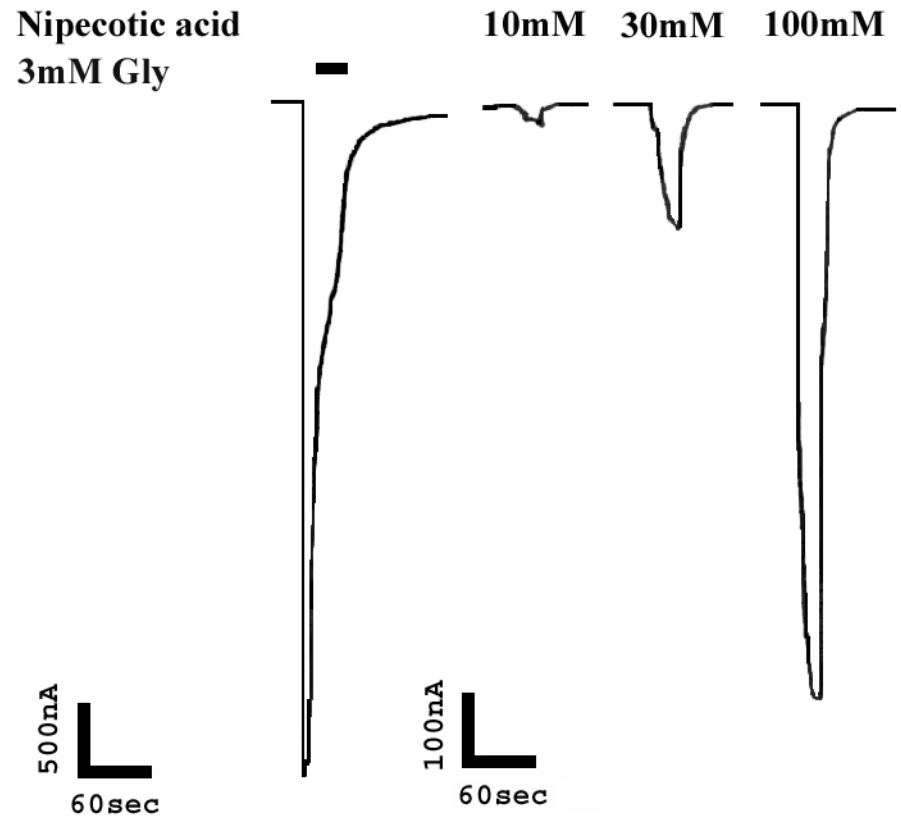


Figure 4.2 Nipecotic acid acts as a weak partial agonist at the α1 D97R/R131D double mutant Tracing of currents demonstrating the gain of efficacy of the competitive antagonist nipecotic acid at the reversal of charge mutant D97R/R131D.

4.3.2 Reversal of charge in the α1D97R/R131D double mutant restores receptor closed state stability

Both the single α1D97R and α1R131D mutations result in receptors exhibiting some levels of spontaneous activity (leak current in the absence of an agonist). As we had previously done with the swap charge reversal between the α1D97 and α1R119 positions, we used the reversal of charge at the double mutant α1D97R/R131D to study whether this could restore GlyR closed state stability. The double mutant was characterized and wt-like holding currents were observed. The double, reversal of charge mutant, also exhibited no increase in background current when compared to the single mutants α1D97R and α1R131D, and was at levels that resembled wt α1GlyR (**Figure 4.1b**). These results are consistent with the model in which α1R131 is in proximity to α1D97, which allows electrostatic interactions between these two residues to occur. This bridging appears to be necessary for keeping the receptor in more stable closed state.

The glycine concentration-response curve was unchanged in this double mutant, compared to wt α1 GlyR, and taurine still remained a full agonist, often exhibiting even higher maximal currents than those induced by maximally-effective concentrations of glycine (**Figure 4.3**).

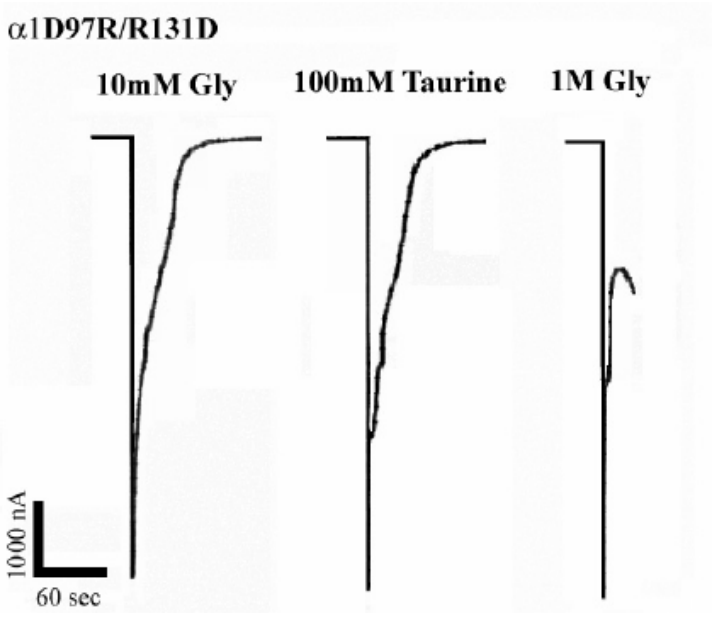


Figure 4.3 Representative tracing showing an increase in taurine potency in the double mutant α1 D97R/R131D Here we demonstrate the change of taurine from a partial agonist into a full agonist at GlyRs carrying this charge of reversal double mutation even though on the whole cell level the stability of the closed state has apparently been reinstated.

4.3.3 α1D97C and α1R131C residues are in proximity and in the right orientation to interact and form disulfide bonds

In order to examine whether these residues are in the right orientation and close enough to interact, we engineered cysteines residues in positions α1D97 and α1R131. Glycine responses measured on this double mutant D97C/R131C α1 GlyRs were stable, but low, suggestive of disulfide bond formation and consequently suppressed receptor activation. The reducing agent DTT was applied to these mutants to break apart the disulfide bonds that we hypothesized would form if these two engineered cysteines were in proximity and in the right orientation to interact. We saw that reduction by DTT successfully increased the glycine response but also elevated the holding current, presumably because receptors lacking this disulfide bond were more likely to spontaneously open (**Figure 4.4**). As seen in the representative tracing, this leak current decreased with each successive glycine application. This decrement in current over time was most likely occurring due to the spontaneous reformation of disulfide bonds as a result of the movements of GlyR binding interfaces in response to the binding of glycine.

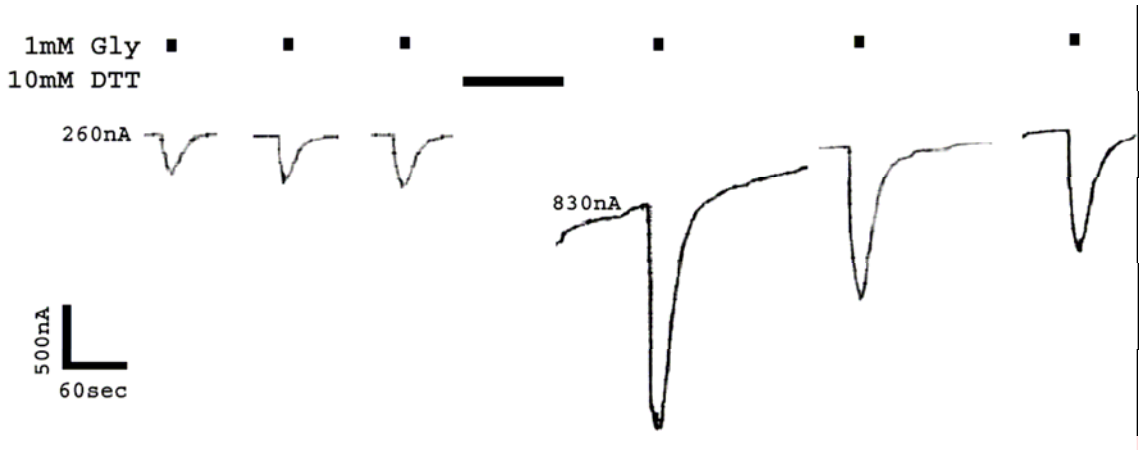


Fig 4.4 Disulfide bond in α1 D97C/R131C can be reduced with DTT Representative tracing showing that at least some receptors with a double mutation form disulfide bonds that suppress channel activity. It should also be noted that after the reduction with DTT not only is an increase in glycine response observed but also an increase in the holding current, highlighting the fact that, when this inter-cysteine bond s broken, the closed state is destabilized once again. Numbers in tracings represent the holding currents at the times indicated.

4.3.4 In D97R/R131D α1 GlyR taurine still acts as a full agonist

Reversal of charge in the double mutant α1D97R/R131D eliminates spontaneous channel-opening activity. We believe that, as in D97-R119, restoration of the attractive electrostatic force that is lost in α1D97R or α1R131D single mutants causes the binding interface of adjacent subunits to come closer together. When this attractive force is re-established this holds the binding domain together and prevents transduction of a channel-opening signal to the gate when the agonist is not present. As seen in the representative trace in Figure 4.3, taurine possesses full efficacy on the double mutant α1D97R/R131D.

4.4 Discussion

Data presented in chapter 3 illustrated how an inter-subunit electrostatic interaction plays a critical role in stabilizing the receptor in its closed channel state. From updated structural models of GlyR generated by Dr. James Trudell (Stanford Univ.) other residues were identified that could potentially contribute to this interaction. We found that arginine at position 131 can indeed form a salt bridge with D97 in α1 homomeric GlyR. On a whole cell level, when re-instated in the double mutant, this interaction re-stabilizes the receptor's closed state. This is represented as decrement of the background current seen in the single mutants and the lack of measurable direct effect of PTX, or 200mM EtOH **(Figure 4.1b and 4.3).**

It is interesting to note that taurine, in most cases a partial agonist at wt GlyR, was converted into a full agonist at both D97R (Welsh et al., submitted) and R131D mutant receptors. This may indicate a significant role of this region for the mechanism of channel activation by both full and partial agonists. In particular it has been hypothesized that partial agonists have decreased efficacy because their binding yields a diminished capacity of transferring the receptor to the flipped closed-channel state that precedes channel opening. After the closed to flipped conformational change is accomplished, glycine and taurine should, by this proposed mechanism, activate GlyR to the same extent (Lape at al., 2008). This is how Welsh et al. (submitted) proposed taurine became a full agonist on α1D97R GlyR. The weakened bond between α1D97R and α1R119 primes the receptors in such a way that they can attain the flipped state in the absence of

agonist, allowing the flipped to open transition to occur as if receptors have already passed the first energy barrier of the transduction process. It was then logical to expect that restoring this electrostatic interaction should also return taurine efficacy to that of the wt GlyR. This proved more complicated to test due to the dramatically rightshifted nature of the glycine concentration-response curve in the α1D97R/R119E double mutant. They were able to use the fact that nipecotic acid, normally an antagonist at GlyR, converts into a partial agonist at α1D97R. Nipecotic acid regains its antagonistic properties when the bond is restored in the double mutant α1D97R/R119E. When I found that another amino acid could interact with α1D97, in this way stabilizing the closed channel state I decided to revisit this issue and test taurine and nipecotic acid efficacies on this double mutant. The reversal of charge in the double mutant α1D97R/R131D produced maximally attainable currents comparable to those of wt GlyRs (at the same ranges of glycine concentrations). The stabilization of the closed channel state in the double mutant did not reverse taurine or nipecotic acid actions at α1D97R/R131D. Even though this double mutant appears to stabilize the closed state on the whole cell level we cannot conclude with certainty that there are absolutely no brief openings occurring in these receptors in the absence of the agonist. The α1D97R/R131D electrostatic interaction may not be as strong as that seen in wt GlyR. Due to the limits of whole cell electrophysiology this would not be detected in our experiments, as we have seen previously in the case of the α1D97R/R119E double mutant. They displayed brief spontaneous openings that were detected in single channel recordings, although tonic current was not detected in whole-cell recordings. Nevertheless, the fact that both α1D97

and α1R131 mutations cause decreased stability of the closed state, have been implicated in the formation of the binding pocket, and seem to be at a junction point early in signal transduction where efficacy can already be influenced, we can conclude that this region plays a significant role in controlling GlyR function.

Overall these data suggest that the residues studied here play a critical role in stabilizing the closed state of the receptor. This stabilization may occur by a simple one-to-one interaction between α1D97 and α1R131 but it is much more likely that other charged residues that constitute this domain contribute as well. As we have previously seen (**Chapter 3**) an inter-subunit salt bridge between α1D97 and α1R119 successfully re-established the integrity of the closed state. In this chapter we add to the understanding of the character of the interface critical for proper GlyR function by showing that the α1R131 residue can interact with α1D97 as well. We cannot conclude with certainty which pair of residues interacts to a greater extent in the wt GlyR. It could be that, as we proposed earlier, these residues form a cloud of charges, necessary in its entirety for the normal functioning of the α1 homomeric GlyRs. It is safe to conclude that this area certainly holds the key to better understanding many aspects of the connection between the binding of the agonist and subsequent proper gating of the channel.

CHAPTER 5: AN ANALYSIS OF THE STRUCTURE OF THE UPPER PORTION OF THE α1 GLYR TM2 SEGMENT IMPORTANT FOR SIGNAL TRANSDUCTION AND ALLOSTERIC MODULATION

5.1 Introduction

The glycine receptor is a pentameric protein characterized by high sequence homology with other members of the cys-loop receptor superfamily of subunits. It is evident that these ion channels, that exert their actions by selective ion permeation across the membrane, share a number of secondary structural features as well. All of the members of the family are integral membrane proteins. This, together with low availability and difficulties inherent in the crystallization of membrane-bound proteins makes structural studies a challenge. Most of our knowledge of GlyR structure comes from pioneering research conducted on the nAChR and nAChBP. Although comparative studies and use of homology modeling using the available nAChR crystal structures and GlyR sequence has contributed a great deal of valuable information, it is important to remember that these proteins are characterized by some fundamental differences as well.

Notably, when comparing GlyR and nAChR, they are activated by different agonists, their function is modulated by different compounds, and they are selectively permeable to different ions. These functional differences must be due to fundamental

variations in structure. In 2005 Unwin published a study describing details of the high resolution nAChR cryo electron microscopy (cryo-EM) structure. At 4 Å resolution this study of the nAChR structure gave us a more detailed view of distinct receptor domains and how they are arranged with respect to one another. His work has since been used extensively to make conjectures about structurally related receptors. Unwin's study reported that TM2, the pore forming domain, was fully alpha helical (Unwin, 2005). This would mean that the side chain of about every third to fourth residue contributes to the formation of the pore region while the rest are pointing in toward the other TM segments or adjacent subunits. This was consequently studied in GlyR but different conclusions were reached. Work by Ma and colleagues indicated that the helical structure of the TM2 domain extends to Q266 (Q14') and possibly to S267 (S15'). Their results were obtained from NMR studies of truncated TM2 and TM3 domains from the α1 GlyR. It is also important to note that in their studies, they used trifluoroethanol (TFE) for the exploration of the structural stability and dynamics of the TM2 region. TFE is known to support the formation of alpha helical structures (Ma et al., 2005). We report here, from our electrophysiological studies on the homomeric α1 GlyR that the upper, more extracellular segment of TM2 (T265-S270; T13'-S19') appears to be characterized by much higher levels of flexibility than could be accounted for if this section was constrained as a true alpha helix. Cysteine substitutions and disulfide bond formation experiments led us to conclude that these residues can cross-link between the subunits. In this way our results agree with the published NMR results detailing the secondary

structure of the TM2 domain (Ma et al., 2005) and with the GLIC structure model (Bocquet et al., 2000).

5.2 Methods

General methods, including primer design, site directed mutagenesis, oocyte isolation and injection, and electrophysiological procedures were described in Chapter 2. Methods not previously used are outlined in more detail below.

To determine the disulfide bond formation in each of the engineered mutant receptors (T265C, Q266C, S267C, S268C, G269C and S270C) maximally effective glycine concentrations were identified for each mutant.

After two or more applications of glycine, the reducing agent, DTT, was applied for 3 min at a concentration of 10 mM. After a 10-12 minute washout period, glycine applications were repeated (at the initial saturating glycine concentrations) two or three times, to determine if the reducing agent had any effect on GlyR function. In the same oocyte, mutants were tested with the oxidizing reagent H_2O_2, applied to favor the formation of disulfide bonds between free sulfhydryl groups; this would occur if cysteine side chains were in close enough proximity and in the right orientation to interact. In some of the preliminary experiments the cross-linking agent $HgCl_2$ was used as well. The application of this compound followed the same protocol as the H_2O_2 oxidation reaction. $HgCl_2$ was applied for a minute at a concentration of 10 μM, and possible changes in function of mutant and wt GlyR were assayed after a 7-12 minute washout. During the applications of reducing, cross-linking or oxidizing reagents, oocytes were unclamped, and both voltage and current electrodes were removed from the bath to minimize

exposure of the glass electrodes to the bath solutions. Oocytes expressing wild type receptors were previously tested for any changes that might arise from re-impaling the cells with electrodes and no significant differences in currents were recorded (data not shown).

5.3 Results

5.3.1 T265C to S270C single mutant substitutions all spontaneously cross-link across subunits.

In order to assess whether individual cysteine substitutions from threonine 265 to serine 270 would affect GlyR function we first determined the concentrations of glycine producing maximal effects. In all mutants we observed a significant decrease in maximally attainable currents (consistent across the cells we tested) compared to maximal currents elicited in wt GlyR. We then investigated whether this decrease could be reversed following the application of the reducing agent DTT. After the initial two or three applications of 10mM glycine, DTT and H_2O_2 or $HgCl_2$ were applied to probe for changes in receptor function. We recorded significant increases in current after 3 min applications of 10 mM DTT in T265C, S267C, S268C, G269C and S270C α1 GlyR **(Figure 5.1a and Figure 5.2).** In contrast, current levels decreased considerably in all mutants tested, when they were exposed to 0.03% H_2O_2 or 10 μM $HgCl_2$ **(Figure 5.1 a and Figure 5.2)**. Both DTT and H_2O_2 were tested for possible effects on wild type α1 GlyR and were without effect **(data shown for DTT in Chapter 3: Figure 3.7a).** This wt control has been tested previously by others (Lobo et al., 2004, Dupre, personal communication) and just confirms that in the wild type α1 GlyRs no cysteine residues are free to interact with one another, and those that form disulfide bonds (as is case with the four cysteines forming the two cys-loops in the ECD) are not accessible to DTT.

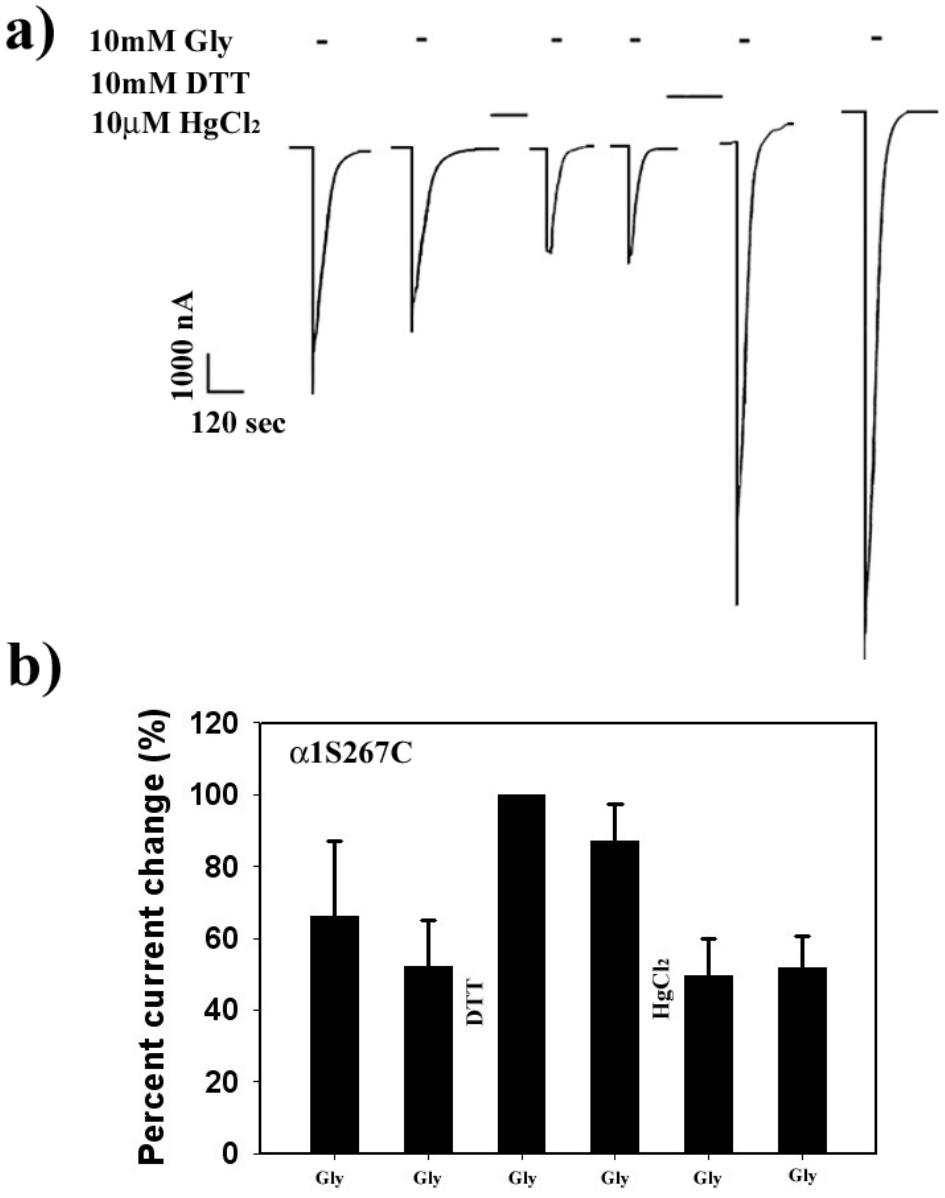


Figure 5.1 The α1 S267C glycine receptor forms inter-subunit disulfide bonds
a) Tracings of currents recorded from homomeric S267C α1 GlyR, demonstrating the occurrence of crosslinking at this mutant receptor. In this case the crosslinking reagent $HgCl_2$, causes a decrease in current while reduction with DTT significantly increases glycine-induced currents at these receptors. **b)** Bar graph summarizing the data recorded from α1 S267C GlyR before and after exposing oocytes to reducing and crosslinking agents. From left to right the vertical bars represent consecutive applications of 10 mM glycine and the applications of DTT or $HgCl_2$ in the absence of glycine are also noted. Data are presented as mean ± SEM of 4-6 oocytes.

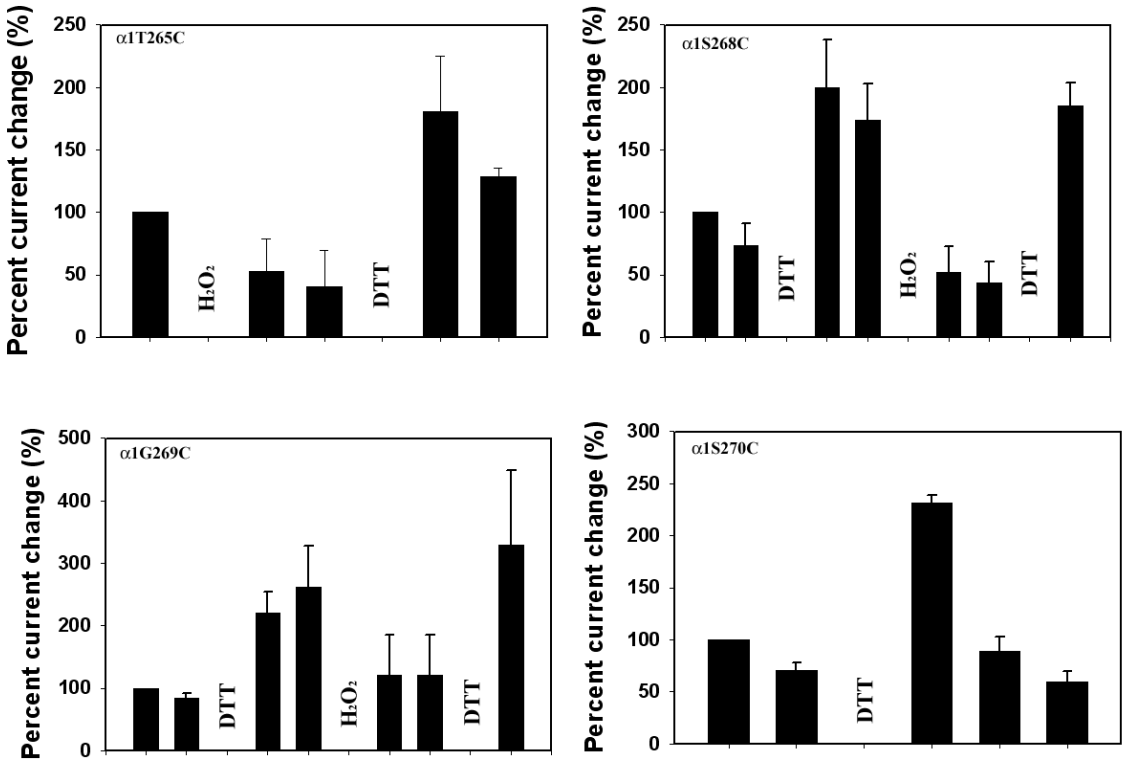


Figure 5.2 Inter-subunit disulfide bonds form between residues of the upper TM2 segment. Summaries of results obtained from receptors bearing cysteine substitutions at positions T265 (upper left), S268 (upper right), G269 (lower left) and S270 (lower right). In each case, from left to right the vertical bars represent consecutive applications of 10 mM glycine; in addition the applications of DTT or H_2O_2 in the absence of glycine are also noted. These bar graphs clearly demonstrate that crosslinking occurs in these receptors, leading to decreases in current responses to maximally effective glycine concentrations after the oxidation and increases in currents (reversal of the effect of disulfide bond formation) after reduction with DTT. The data shown represent the mean ± SEM of 3-5 oocytes.

5.3.2 A special case of cross-linking at α1 Q266C mutant receptors

In the case of the α1 Q266C mutant we obtained different results depending on the order of events in the oxidation-reduction protocol. These receptors showed a progressive decrement in current as a result of recurring glycine-induced activation **(Figure 5.3 a).** When DTT is applied first this results in a significant increase in glycine current. When, in the same oocyte, this is followed by an application of H_2O_2, this results in further current increase **(Figure 5.3 a and b).** However, different results are obtained when the order of application of the oxidizing and reducing agents is reversed. When application of a maximally-effective concentration of glycine is followed by an application of H_2O_2 before the oocytes have been exposed to the reducing agent, this results in a significant increase in current **(Figure 5.3 b).** The subsequent application of the reducing agent then, either caused a small reduction, or an increase in the glycine-evoked currents **(Figure 5.3 b).**

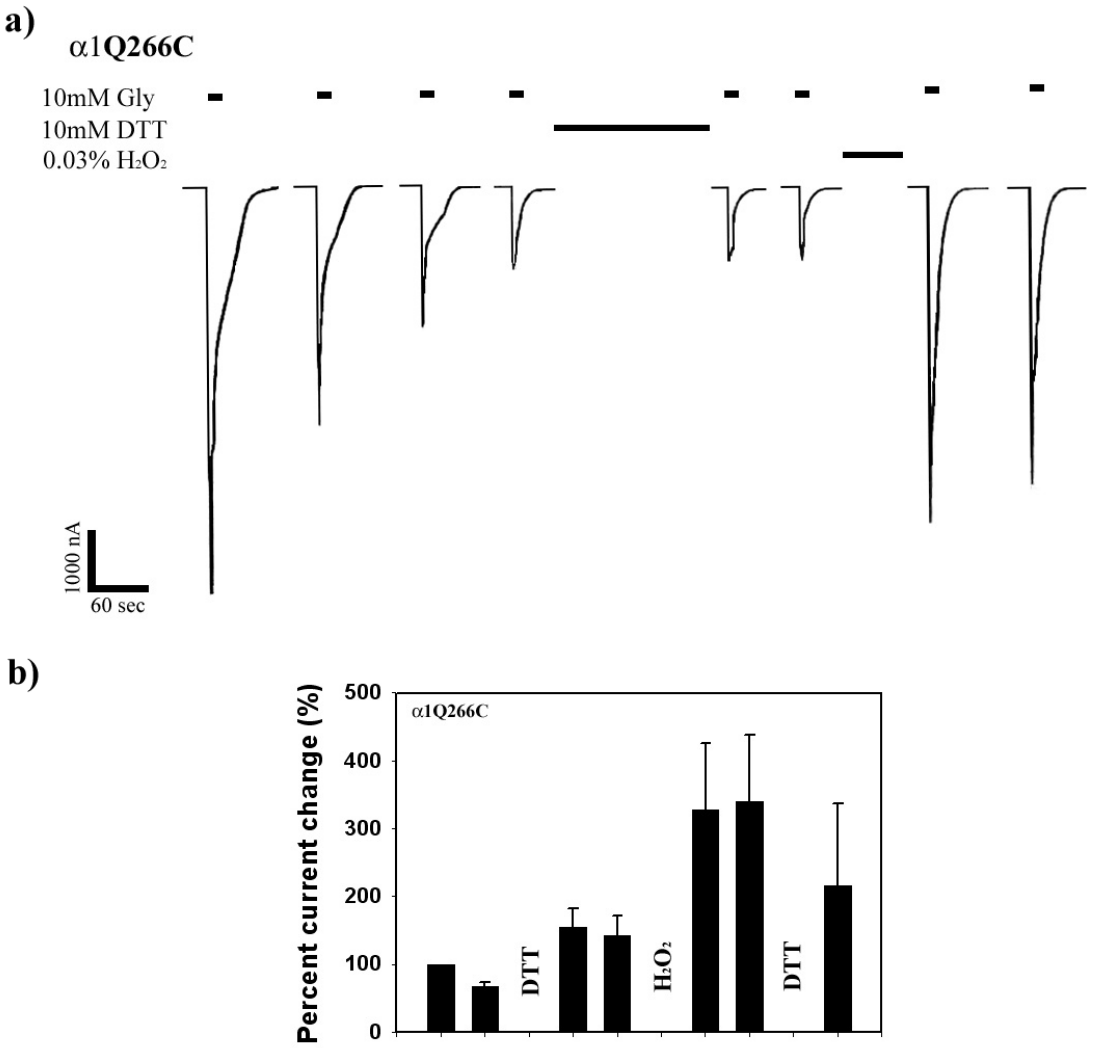


Figure 5.3 An unusual case of crosslinking at the α1 Q266C mutant
a) Sample **t**racing showing one of the effects of disulfide bond formation at Q266C. The decreases in glycine-mediated currents due to recurrent glycine applications are evident. In this case the reducing agent DTT did not appear to have an effect while application of H_20_2 increased glycine-induced currents significantly. This DTT effect was an unusual but reproducible finding seen in only some oocytes. **b)** On average the reducing agent (when applied first) had the expected effect of enhancing subsequent glycine-induced currents. Application of the oxidizing agent H_2O_2 had unexpected results, increasing glycine currents in all oocytes tested. After the oocytes were exposed to H_2O_2 a second application of DTT caused either a decrease in current or had no apparent effect on these receptors. Data are presented as mean ± SEM of 3-7 oocytes.

5.3.3 Rates of cross-linking in the S267C α1 GlyR is affected by channel activation

We determined whether differences exist between the rates of cross-linking with or without re-applications of the agonist; i.e., does spontaneous cross-linking occur and can the rate of cross-linking be accelerated by receptor activation? We applied DTT for 3 min and after a 7-10 minute washout glycine was then applied either four times at 12 minute intervals or once after a 48 minute washout period. The final measurements of current levels were then compared to determine the dependence of cross-linking on movement of this segment during channel activation **(Figure 5.4).**

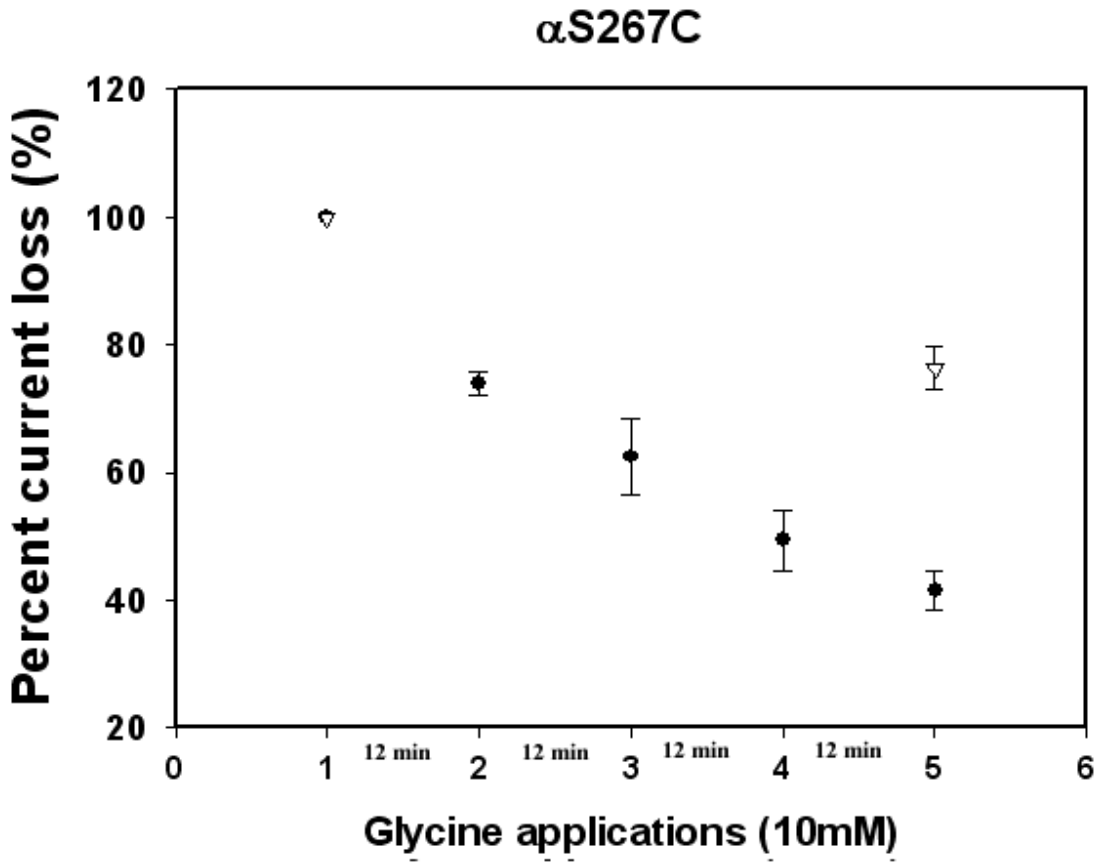


Figure 5.4 The rate of crosslinking at S267C depends on receptor activation. A scatterplot graph demonstrating the dependence of rates of crosslinking at α1 S267C GlyR on glycine application. Filled circles represent crosslinking as it occurs when max glycine is applied every 12 min, bringing the current to 41.4 ± 3.1% of the initial glycine response. Triangles represent the percent current loss when maximal glycine was applied only once 48 minutes after the initial glycine application. This results in a significantly smaller decrement in the glycine-evoked current (to 76.4 ± 3.3% of initial current). Data are presented as mean ± SEM of 3-5 oocytes.

5.3.4 S267C does not interact with free α1 GlyR cysteines at positions 41 and 290

In the wild type α1 GlyR three free cysteines could potentially interact with another cysteine residue if it was in the right orientation, environment and proximity. These cysteines are all positioned far from the segment that we studied here. C41 is located in the extracellular domain, C290 is in TM3, while C345 is situated intracellularly, as part of the cytoplasmic TM3-4 loop. We considered the possible interaction of C345 with S267C as highly unlikely, and did not consider it further. We tested the possible interactions of S267C with C41 or C290 by engineering the double mutants α1C41A/S267C (data not shown) and α1S267C/C290A **(Figure 5.5).** These double mutants yielded results that were the same as those obtained from the α1S267C single mutant. A decrease in current as a result of a disulfide bond formation was still evident and reduction and oxidation reaction results were comparable to those seen in the single α1S267C mutant. We can therefore conclude that the naturally occurring cysteine residues cannot interact with S267 residue **(Figure 5.4).** Although we still have to test the remaining residues that we studied here, we at present assume that they would also not be affected by these free cysteine residues. This is something that can be done in the future to ensure no interaction is possible between these TM2 amino acids and either C41 or C290.

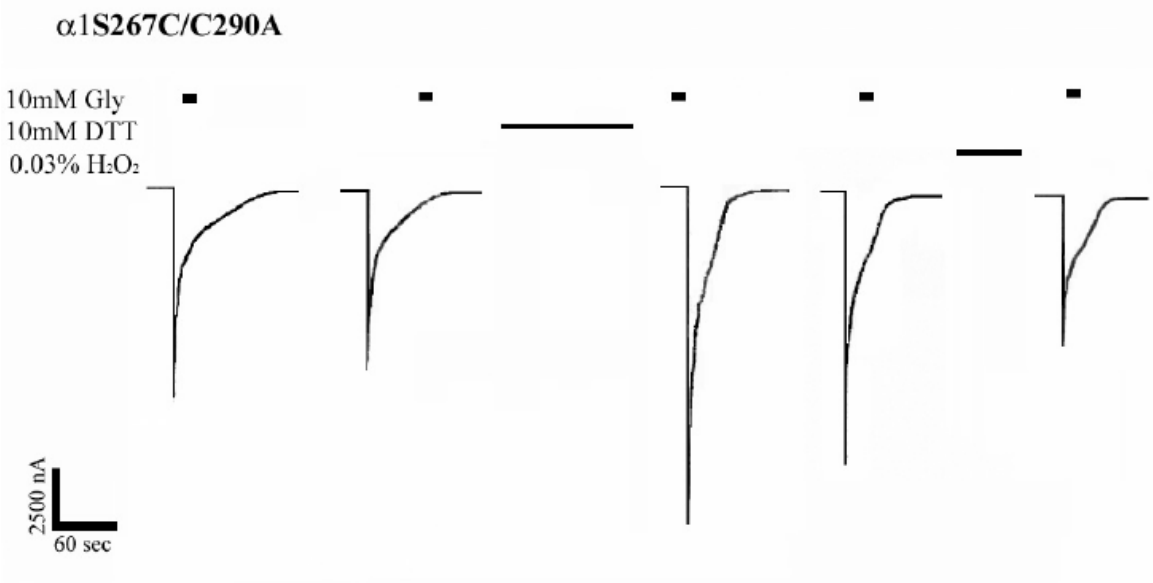


Figure 5.5 S267C does not crosslink with the endogenous cysteine residue at position C290 in TM3. Representative tracing of currents recorded from the double mutant S267C/C290A, demonstrating that when the C290 residue is mutated to alanine, the disulfide bond formation observed in the α1 S267C mutant GlyR remains conserved.

5.3.5 Maximal current at S267C is increased by PMTS

Previous work from our lab demonstrated, using single channel recordings, that the α1 S267C mutation significantly reduces the unitary channel conductance (Goldstein et al., in preparation). Wild type-like conductance was re-established after an application of the thiol reagent propyl methanethiosulfonate (PMTS) but what was responsible for this was at the time not further evaluated. Considering that our whole cell measurements showed spontaneous cross-linking at S267C GlyR we hypothesized that PMTS may be breaking the disulfide bonds that formed spontaneously between receptor subunits and relief of this crosslinking could then explain the changes in unitary conductance. To test this further we decided to measure changes in whole-cell maximal currents following the application of PMTS. One would hypothesize that they should be greater if conductance increased due to a PMTS-induced breakage of inter-subunit bonds. After exposing the cell to the concentrations of glycine (10 mM) that induced a maximal current in the α1 S267C mutant, we applied 50 μM PMTS for 1 minute. Following a washout period of 10-12 minutes the same glycine concentration was re-applied and a significantly greater current was observed **(Figure 5.6).**

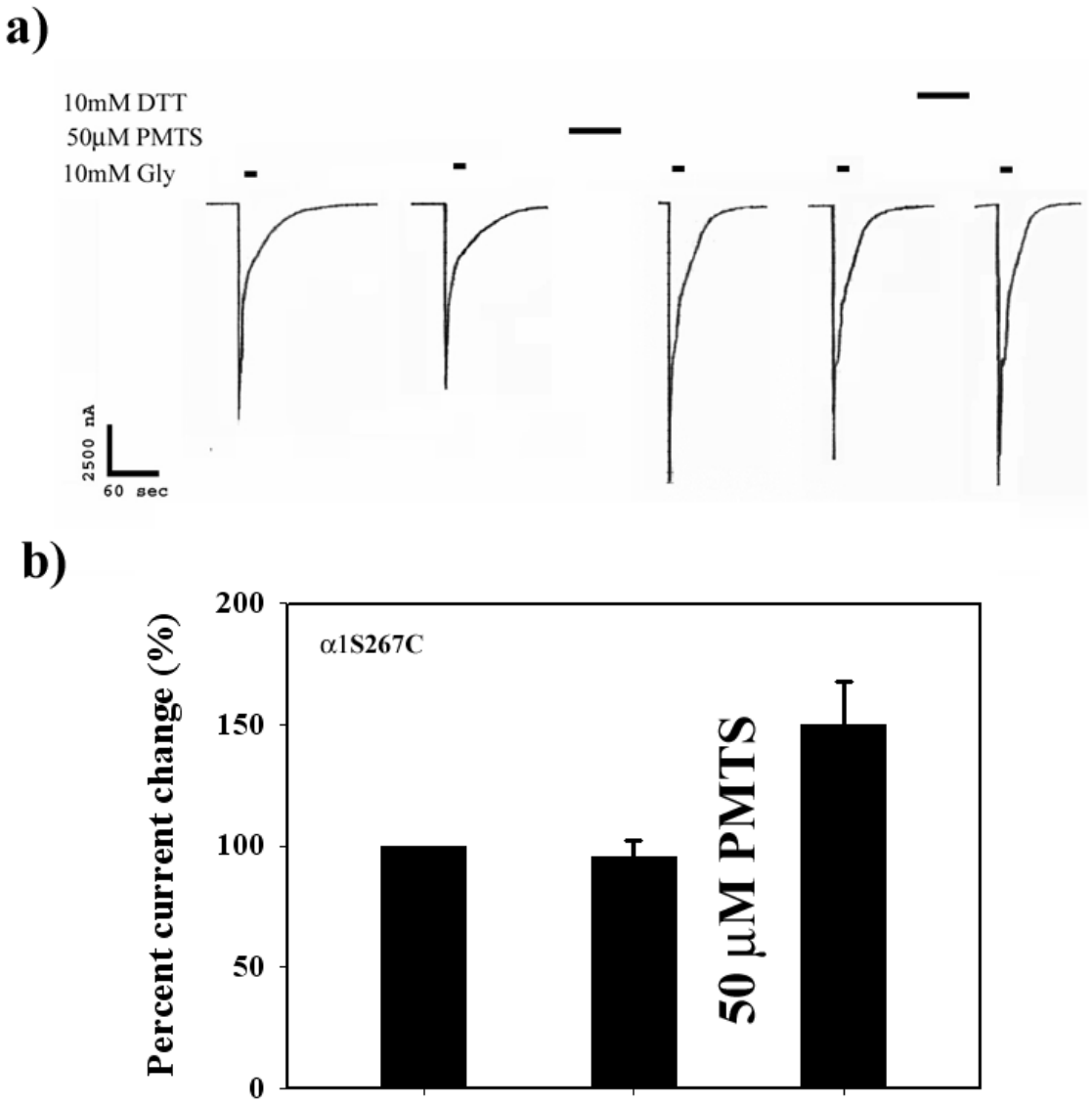


Figure 5.6 PMTS increases maximal glycine currents at S267C mutant receptor.
a) Representative tracings illustrating the increases in glycinergic currents at α1 S267C GlyR after the receptors were exposed to 50 μM PMTS. **b)** Bar graph showing the average increase in current recorded from the S267C mutant receptors after exposure to PMTS. The left and middle bars represent currents induced by a maximally effective glycine application before PMTS has been applied, while the right bar represents currents evoked by the same receptors after PMTS exposure. Data are presented as mean ± SEM of 11 oocytes.

5.4 Discussion

My research demonstrates that the upper segment of TM2, the pore lining segment, does not appear to be constrained in a true alpha helical shape. If the secondary structure of this segment were truly helical it seems unlikely that the engineered cysteines we evaluated could all interact with each other across the interface of two adjacent subunits. The flexibility of this region may indeed play an important role in both the activation and modulation of GlyRs. Although differing in the degree of disulfide bond formation, T265C, Q266C, S267C, S268C, G269C and S270C α1 GlyR can all spontaneously cross-link with the same residue on an adjacent subunit. This may not be surprising if we consider that this segment feeds into the TM2-3 linker region that is believed to play an important role in transferring the binding signal from the ECD to the pore region and possibly even between the subunits (Dupre et al., 2007). The flexibility of this segment may have additional implications in the dynamics of signal transduction. Positions Q266 and S267 may be of particular interest considering their importance in GlyR function. Among the identified mutations causing hyperekplexia, a missense mutation at position 266 is the only mutation occurring within TM segments (Milani et al., 1995). Moorhouse et al. (1999) showed that a histidine for glutamine exchange at position 266 in the TM2 domain leads to destabilization of the channel open state. This mutant, however, does not affect the receptor's sensitivity to Zn^{2+} (tested due to the high affinity interaction of Zn^{2+} with histidine) or pH changes: this, they thought, hinted at the possibility that the H266 side chain is facing away from the channel lumen. In addition,

their data demonstrated that this mutation produces a decrease in taurine's efficacy, making it a weak partial agonist at these receptors (Moorhouse et al., 1999). Another group, working to solve the structure of the GlyR TM2 segment, looked into Q266H substitution and concluded that histidine at this position may be preventing normal gating movements by stabilizing anions in the pore due to the positively charged histidine side chain (Tang et al., 2002), suggesting that this could explain why Q266H spent significantly less time in the open state than the wild type α1GlyR (Moorhouse et al., 1999). Furthermore, their NMR structure studies indicated that this Q266 residue at the 14' position forms the most constricted part of the pore (Tang et al., 2002). This would agree with the inconsistent data that we obtained with the reduction and oxidation protocols on this mutant. It could be that Q266C can cross-link not only between adjacent subunits but perhaps also across the pore, giving us in this way differing results after DTT and H_2O_2 application. This may be why, depending on the order of application of reducing and oxidizing agents, we measured increases in current after the application of DTT, and reductions in current when H_2O_2 was applied following reduction. The same applications, but occurring in a different order, with oxidation first and reduction second, resulted in unexpected results. When receptors were first exposed to the oxidative environment this resulted in an increased current. This was followed with either a small reduction or a further increase in current following the application of DTT. Essentially, under the two scenarios, we could be dealing with different populations of receptors. Some may have cross-linked between adjacent subunits and others across the pore, giving us in this way different outcomes from, in principle, the same protocols.

CHAPTER 6: GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

Ever increasingly sophisticated electrophysiological procedures and advances in LGIC structural models have provided great insight into GlyR function in the last decade. The activation processes of these proteins have proved difficult to decipher, and studies of this nature still represent an enormous challenge. Most of the current knowledge about the processes of binding and signal transduction of the GlyR is based on pioneering work done on the nAChR and nAChBP. Although advances made in our understanding of the structures of these related receptors provided a wealth of clues to aid in interpreting the puzzle that is the GlyR one must always remember the caveat that the protein subunits in this superfamily share less than 20% primary sequence homology.

The research described in this dissertation delineates some of the critical processes necessary for the proper functioning of the GlyR, in this way furthering our understanding of these ion channels.

6.1 Significance of ion channel closed state stability in the absence of the agonist

The proper functioning of the GlyR and other ligand-gated ion channels (LGICs) depends on ligand binding initiating the process of signal transduction. The presence, timing, and the concentration of different agonists that are released onto a postsynaptic cell represent one of the important controls of neuronal communication. Many subsequent events such as action potential generation and neurotransmitter release rely on the proper signaling that is initiated at LGICs. The neuronal connections and networks that they help form are like a finely tuned instrument: if one of the strings breaks, the music just doesn't sound the same.

Although examples of tonic activity in wildtype receptors exist, these occurrences of spontaneous channel openings are rare, and among cys loop receptors, have only been seen in the nicotinic acetylcholine receptor (Jackson, 1986). Glycine receptors that may at times appear to open spontaneously are instead displaying currents simply because sufficiently high and persistent concentrations of agonists such as taurine or β-alanine are tonically present, such as at extrasynaptic GlyR. Thus far no evidence exists that any wildtype GlyR can form channels that activate spontaneously in the absence of an agonist. In Chapter 3 I studied a mutant GlyR involving an aspartic acid substitution at position 97 in the ECD that confers tonic channel opening activity. This was characterized by a strychnine-sensitive leak current that was observed even when glycine was absent from the bath solution. Some of the receptors in this population pool were in

the closed channel state and were still activatable by glycine. This conclusion was reached after examination of α1 D97R single channel data that demonstrated that the probability of these channels being found in the open state in clusters in the absence of the agonist was comparable to that of wildtype α1 GlyR exposed to saturating glycine concentrations (>90%). The magnitudes of whole-cell inward currents elicited by glycine in the α1 D97R mutant cannot be explained simply as increases in channel Po and therefore this suggest that a subpopulation of channels is found in a closed and activatable state. In other words, the α1 D97R mutation does not force channels into an open state; instead it increases the likelihood that they will transition from a closed to an open state, in the process promoting the possibility of cluster initiation. Once the cluster has been initiated, the channel seems to behave much like a wildtype receptor exposed to a maximally effective glycine concentration. In a study performed by Beckstead et al. (2002) the phenomenon of spontaneous receptor openings seen in the D97R mutant was used to further delineate the mechanisms by which modulators such as alcohols and volatile anesthetics affect GlyR function, but major questions remained following that study. The Todorovic et al (2010) paper helps to clarify remaining issues. In two-electrode whole-cell studies conducted on α1 D97R GlyR expressed in oocytes Beckstead et al (2002) found that ethanol enhanced tonic channel opening but did not enhance any currents elicited by even low concentrations of glycine on this mutant; i.e. the effects of ethanol and glycine appeared additive on α1 D97R receptors. This was quite puzzling until we showed that tonically opening α1 D97R GlyR behave as though they have bound a maximally effective glycine concentration (P_o >0.9). One would not

expect ethanol to enhance the function of receptors with a Po this high since ethanol does not affect P_o (Welsh et al, 2009). Instead ethanol could be acting by increasing the likelihood of cluster initiation, antagonizing desensitization (leading to longer clusters) or increasing the rate of transitions between desensitized and closed states. The last two possibilities of the three seem unlikely since ethanol does not affect currents elicited by long-term exposure to a saturating glycine concentration in wt α1 GlyR, at which time channels would be expected to be in equilibrium between desensitized and open states (Welsh et al. 2010).

These data also explain the very low Hill slope number (0.61) found by Beckstead et al. (2002) for α1 D97R glycine concentration-response curves. In wildtype α1 GlyR the Hill slope is typically around 1.5-2, indicating cooperativity of binding as multiple glycine molecules sequentially bind to a receptor. The loss of cooperativity in the α1 D97R GlyR makes sense when one considers that spontaneously-active receptors have Po values almost identical to those of maximally-activated wildtype receptors that have bound as few as three glycine molecules (Beato et al. 2004). If spontaneously-activating receptors result in clusters of high Po activity one would expect the same to be true of clusters initiated by the binding of even a single glycine molecule. As a result the glycine concentration-response curve in the α1 D97R mutant reflects just the likelihood of binding and consequent channel activation, with no cooperativity evident.

6.2 Inter-subunit electrostatic interactions play a significant role in GlyR function

The research described above identifies several different residues that can interact with aspartate-97 to stabilize the closed-state of the GlyR in the absence of agonist. It would be overly simplistic to postulate that only one such interaction was possible or even likely since proteins such as the GlyR are not static structures. I concluded that structurally and functionally α1 K116, R119 and R131 can all, to variable degrees, fulfill the role of electrostatically interacting with D97 and thus contributing to the inter-subunit attractive force that helps keep the receptor from spontaneously activating. K116 seems to be the least important of the three since charge reversal experiments involving the double mutant α1 D97R/K116D yield channels that still exhibit significant tonic activity, albeit at levels below those of the D97R mutant. For this reason my effort was largely expended in studying the R119 and R131 residues. In both cases the double charge reversal mutants (α1 D97R/R119E and α1 D97R/R131D) resulted in wildtype-like holding currents suggesting the re-establishment of inter-subunit electrostatic bonds. Cysteine substitution experiments confirmed the physical interactions of residue 97 with cysteines introduced at positions 119 and 131. Interestingly, although the α1 D97R/R119E double mutant appeared to exhibit wildtype-like holding currents when measured on the whole-cell level, single channel recordings showed very brief spontaneous openings. This was not surprising if one considers that even in the charge reversal double mutant, the R97 and E119 electrostatic interactions are not the only

possible interactions in which these two residues participate. For example, arginine replacing aspartate at position 97 would be expected to change the conformation of the – side of the subunit interface bearing that residue in addition to interacting with residue 119 on the + side. The same holds for residue 119 on the + side of the interface in the ECD. It is interesting to speculate if comparable inter-subunit, or perhaps intra-subunit, electrostatic interactions affect open state stability; i.e., are there specific electrostatic interactions involving α1 D97, R119 or R131, or perhaps other as yet unidentified charged residues, that stabilize channel open states?

6.3 Upper TM2 segment structure and implications of its flexibility on GlyR function and modulation

Binding of glycine in the ECD between adjacent subunits is the first step in the activation of GlyR. This binding signal is then transduced to the pore and a number of distinct regions of these receptor subunits are implicated in this transduction process. One of these regions is the extracellular portion of TM2 as well as the TM2-3 linker region. Structural data obtained from studies conducted on nAChR (Unwin, 2005), nAChBP (Brejc et al., 2001), and the GLIC (Bocquet et al., 2009) and ELIC (Hilf and Dutzler, 2008, 2009) channels suggests that these proteins have varying structures in their extracellular portions of TM2. In addition some preliminary studies I conducted on the S267 residue in TM2 suggested that this amino acid may not just play a role in constituting an intra-subunit alcohol binding pocket but also appears capable of sufficient movement to interact with equivalent residues on adjacent subunits. Specifically, when residue 267 is mutated to cysteine it can form covalent cross-links to adjacent C267 residues. If this region in TM2 is truly α helical as the Unwin et al. (2005) model of the nAChR suggests, one would expect only every three or four residues to point in the same direction. Thus not all of the residues in this region should be capable of these inter-subunit interactions upon cysteine mutation. However I found that all of the residues between locations T265 and S270 (upper end of TM2) were capable of crosslinking after cysteine mutagenesis, making it highly unlikely that this region is α helical. This suggests that the structure of the GlyR α1 receptor subunits in this region is closer to that

found in the ELIC (Hilf and Dutzler, 2008) and GLIC prokaryotic proteins (Bocquet et al., 2009) than in the nAChR.

6.4 Overview and future directions

A thorough knowledge of protein structure is important as it can provide us with information of the organization of amino acids relative to one another as well as the local environment of different protein domains. In research on LGICs this can further assist us in elucidating how different agonists and modulators may be stabilized upon binding, giving us more insight into their mechanisms of action. As seen from the research presented above new information obtained regarding the receptor structure may also reveal critical interactions that occur within and between the subunits, in this way helping us to navigate through the details of signal transduction, channel gating and receptor modulation by various endogenous and exogenous agents.

The research presented in this dissertation contributes to our understanding of GlyR structure and function as well as answering some questions that remained from previous studies on these and related receptors. However, many questions still remain and the following suggestions will outline some of the future directions that might prove fruitful.

1. In Chapters 3 and 4, I discussed electrostatic interactions that play critical roles in maintaining receptor closed-state stability and also appear to constitute initial steps in the initiation of signal transduction once the agonist has bound. Mutations of charged residues D97R and R131D, that are part of this region, also resulted in increases in the efficacies of the partial agonists taurine, β–AIBA, β–ABA and even changed the antagonist nipecotic acid into a weak partial agonist (Welsh et

al., 2010). A number of possibilities exist to explain differential sensitivities of partial agonists at LGIC's. Lape et al. (2008) conclude from their work that agonist efficacy is dependent on the ability of a ligand to effect transitions between two closed-channel states: the closed and flipped states. Their hypothesis posits that once the receptor adopts the flipped state, which is hypothesized to occur early in the process of signal transduction, from that point different ligands no longer have differenct efficacies;is not affected; i.e., β/α do not vary among ligands such as glycine and taurine. Welsh et al. (2010) developed on this idea further by conducted experiments that allowed them to conclude that the dramatic increase in taurine efficacy at α1 D97R is consistent with the hypothesis that these receptors exhibit a decreased energy barrier for transitions between closed and flipped states. Another possibility is that partial agonism arises through self-antagonizing properties that some of these ligands may have. Schmieden et al. (1995, 1999) show that, in β amino acids that are found in both the cis and trans forms in solution, the cis isomer act as an agonist while the trans form is an antagonist at the GlyR. This hypothesis is strengthened when we take in consideration that nipecotic acid, which is found only in a trans-form, acts as an antagonist at wildtype GlyR. Research presented in Chapter 4 demonstrates that even though in the D97R/R131D double-mutant assayed on the whole-cell level, the receptor did not appear to open spontaneously taurine remains a full agonist and other β amino acids, as well as nipecotic acid, still exhibit increased efficacy at this mutant receptor compared to wildtype. It seems

imperative that single channel recordings be made from the R131D and D97R/R131D receptors. In addition to determining if spontaneous channel opening was occurring one would also look at the responses to a saturating concentration of taurine. The prediction would be that taurine would have a high P_o, comparable to the effect of a saturating concentration of glycine applied to wild type receptors.

2. The majority of data presented in this dissertation has been recorded from α1 homomeric GlyRs. It is important to see if one could generalize conclusions pertaining to the critical interactions that I showed stabilize the closed state of the α1 receptor to the α1β heteromeric GlyR. This would be of significance since it is the heteromeric receptors that are the most commonly found in neurons in mature animals. While aspartic acid at position 97 (**Chapter 3**) remains conserved in the β subunit, positions equivalent to α1 K116 and α1 R119 are not charged residues in the β subunit (I139 and F142, respectively). Nevertheless, arginine at position 131 on α1 (**Chapter 4**) has a homologous charged counterpart in the β GlyR subunit (R154) that should be tested for its possible electrostatic interactions with the D97 residue in the α1 subunit. In addition R145 in the β subunit (equivalent to α1 R122) should also be examined.

3. In Chapter 5, I studied the upper segment of the TM2 domain and determined, through cysteine scanning, that all of the residues studied expressed some degree

of crosslinking (presumably between subunits). It would be interesting to develop on this idea further to show conclusively that this crosslinking occurs between subunits. In order to do so, one would have to run Western blots of the subunits under reducing and non-reducing conditions. Under non-reducing conditions one would expect dimmers of mutant but not wildtype subunits to be present. In addition all of the mutants studied should also be tested on C41A and C290A α1 GlyR backgrounds that remove the possibilities of crosslinking to endogenous cysteine residues. In the case of the Q266C mutant I believe it is quite possible that it may be interacting with C290 because the results of the oxidation experiment were quite unexpected. Considering that a significant increase in the glycine-induced current was observed after receptors were exposed to the oxidizing environment (preference for disulfide bond formation when sulfhydryl groups of free cysteines are close enough and in the right orientation to interact) one could postulate that there may be interactions taking place between C266 and a naturally-occurring cysteine at position C290 of TM3 domain, either within the same subunit or possibly between subunits. After determining whether this may be occurring with the double mutant Q266C/C290A, in the case of a positive result (change in oxidizing reaction effects when compared to the single mutant Q266C: decrease or no effect in the C290A background), one would then differentiate between intra- and inter subunit interaction using Western blotting.

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