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MOLECULAR TARGETS AND MECHANISMS FOR ETHANOL ACTION IN GLYCINE RECEPTORS

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Abstract

Glycine receptors (GlyRs) are recognized as the primary mediators of neuronal inhibition in the spinal cord, brain stem and higher brain regions known to be sensitive to ethanol. Building evidence supports the notion that ethanol acting on GlyRs causes at least a subset of its behavioral effects and may be involved in modulating ethanol intake. For over two decades, GlyRs have been studied at the molecular level as targets for ethanol action. Despite the advances in understanding the effects of ethanol *in vivo* and *in vitro*, the precise molecular sites and mechanisms of action for ethanol in ligand-gated ion channels in general, and in GlyRs specifically, are just now starting to become understood. The present review focuses on advances in our knowledge produced by using molecular biology, pressure antagonism, electrophysiology and molecular modeling strategies over the last two decades to probe, identify and model the initial molecular sites and mechanisms of ethanol action in GlyRs. The molecular targets on the GlyR are covered on a global perspective, which includes the intracellular, transmembrane and extracellular domains. The latter has received increasing attention in recent years. Recent molecular models of the sites of ethanol action in GlyRs and their implications to our understanding of possible mechanism of ethanol action and novel targets for drug development in GlyRs are discussed.

Keywords

Glycine; Ethanol; Targets; Electrophysiology; Molecular Models; GLIC

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1. INTRODUCTION

A. Background

Alcohol abuse represents a significant problem in our society, affecting about 17.6 million people in the United States alone (Volpicelli, 2001; Grant *et al.*, 2004). The economic costs of alcohol-related disorders in the United States are estimated to exceed 185 billion dollars per year (Grant *et al.*, 2004), compared to the 730 million dollars spent on alcohol-related research worldwide (Rajendram and Preedy, 2005). Consumer expenditure on alcohol in the United States in 2001 was 128.6 billion dollars (Foster *et al.*, 2006). The effects of alcohol abuse and dependence result in significant loss of workplace productivity, increased accidents, increased risk for hypertension, cardiomyopathy, obesity, and liver disease. This in turn leads to over 100,000 deaths and costing nearly 200 billion dollars per year (Grant *et al.*, 2004; Harwood, 2000). Despite the wide consumption and the issues associated with the excessive intake of alcoholic beverages, the mechanisms of ethanol action that cause and modulate its behavioral effects are still poorly understood.

Historically, alcohols and anesthetics were believed to act by perturbing the lipid membrane. Over 100 years ago, H.H. Meyer (Meyer, 1899; Meyer, 1901) and C.E. Overton (Overton, 1901) independently found that the potency of alcohols and general anesthetics was proportional to their partition coefficient between the aqueous phase and the oil phase (water and olive oil, respectively) (Roth, 1979; Heimborg and Jackson, 2007). Structural diversity among anesthetic agents lent support to the concept of a nonspecific, lipid target and mechanism of action for all anesthetics (Trudell *et al.*, 1973b; Trudell, 1977). Meyer refined the Meyer-Overton rule (Meyer, 1937) by proposing that anesthesia occurs when any chemically indifferent substance attains a certain concentration within the lipid bilayer of the cell (Roth, 1979). L.J. Mullins supported the notion that anesthetic potency is related to the concentration in the membrane, but suggested that the volume occupied by the anesthetic is also important for anesthesia (Mullins and Gaffey, 1954; Roth, 1979). He predicted that anesthesia occurs when a critical volume fraction of anesthetic exists within the membrane phase. Mullins suggested that the permeability of ions would be depressed once a critical volume of anesthetic within the membrane was reached, thus resulting in a loss of excitability. Later work extended Mullins' theory to suggest that adsorption of an anesthetic would expand the membrane, thereby providing a basis for the mechanism of anesthetic action (Lever *et al.*, 1971; Miller *et al.*, 1973; Halsey, 1982).

The critical volume hypothesis states that "anesthesia occurs when the volume of a hydrophobic region is caused to expand beyond a certain critical amount by the adsorption of molecules of an (inert) substance. If the volume of this hydrophobic region can be restored by changes of temperature or pressure, then the anesthesia will be removed" (Mullins and Gaffey, 1954). This notion was supported by studies showing that exposure to high pressures (>100 ATA) could reverse anesthetic effects of ethanol and other general anesthetic agents (Johnson *et al.*, 1942; Johnson and Flagler, 1950; Lever *et al.*, 1971; Miller and Wilson, 1978; Halsey *et al.*, 1978; Halsey and Wardley-Smith, 1975; Trudell *et al.*, 1973a; Chin *et al.*, 1976; Galla and Trudell, 1980), as well as by more recent studies demonstrating a relationship between body temperature and sensitivity to ethanol at behavioral and biochemical levels (Malcolm and Alkana, 1981; Malcolm and Alkana, 1983; Alkana *et al.*, 1985; Alkana *et al.*, 1988; Finn *et al.*, 1990; Finn *et al.*, 1991).

Similarly, the observation that alcohols and anesthetics protected erythrocytes from hemolysis led to the suggestion that this protection was due to an expansion of the erythrocyte membrane (Seeman and Roth, 1972). These authors suggested that the membrane was expanded isotropically in three dimensions; leading to the proposal that the membrane expansion caused by alcohols and anesthetics was ten-fold the actual volume of those molecules (Seeman,

1972). For several years, this proposal presented a conundrum for molecular mechanisms of intoxication. The realization that a membrane does not expand isotropically, but rather the membrane thins when the surface area expands (Trudell, 1977), resolved this controversy. The latter result suggested that the membrane volume in the presence of alcohols or anesthetics would be only slightly greater than the combined molecular volumes of the ligands plus the original membrane. This concept was shown experimentally by measuring how pressure could exclude small spin-labeled molecules (Trudell *et al.*, 1973a). This notion was codified as a “mean excess volume hypothesis” (Ueda and Mashimo, 1982;Mori *et al.*, 1984). The important point of the “mean excess volume” hypothesis is that, in order to have a response reversed by pressure, the sum of the molar volumes of the agent (alcohol or anesthetic) plus the target (membrane or protein) must be less than the total volume of the new mixed system. This “excess volume” would come about by creating disorder in the system; for example, poorer packing of water molecules in protein cavities or creation of small cavities at the protein-lipid interfaces. Then pressure would act to restore the system to a more ordered lower-volume state. However, the properties of bulk phospholipid membranes were only slightly modified at the pressures that reversed anesthesia (approximately 100 ATA) (Mastrangelo *et al.*, 1978;Mastrangelo *et al.*, 1979;Galla and Trudell, 1980;Galla and Trudell, 1981). As a result, many studies investigated the much more sensitive lateral phase separations in membranes (Trudell, 1977;Galla and Trudell, 1980;Galla and Trudell, 1981).

Convincing evidence indicates that alcohols and anesthetics act on proteins (either within or independent of the membrane) and that membrane perturbation alone is not sufficient to cause anesthesia (Hunt, 1985;Deitrich *et al.*, 1989). The effects of alcohols on membrane disorder are generally measurable only at concentrations well above the pharmacological range (Goldstein, 1984). For example, at concentrations associated with anesthesia, there would be only 1 ethanol molecule per approximately 200 lipid molecules. Furthermore, the effects of intoxicating concentrations of alcohols on membrane order can be mimicked by an increase in temperature of just a few tenths of a degree Celsius (Pang *et al.*, 1980;Franks and Lieb, 1982), which clearly does not produce behavioral signs of alcohol intoxication.

In now classic experiments, Franks and Lieb (Franks and Lieb, 1984) found that general anesthetics inhibit the light-emitting firefly luciferase reaction (a lipid-free protein preparation) and suggested that anesthetics “act by competing with endogenous ligands for binding to specific receptors.” Subsequent work found that highly purified optical isomers of the inhalational general anesthetic isoflurane, which are equally effective at disrupting lipid bilayers, exhibited clear stereoselectivity in their effects on particularly sensitive ion channels (Franks and Lieb, 1991;Jones and Harrison, 1993). Moreover, there was a strong correlation between the stereoselective effects of isoflurane on these ion channels and the potency of the anesthetic isomers *in vivo* (Harris *et al.*, 1992;Lysko *et al.*, 1994).

Collectively, these findings demonstrate that the lipid bilayer cannot account for all of the alcohol and anesthetic effects in the CNS and that more specific sites of action (such as membrane proteins) also play an important role. In spite of the bodies of evidence implicating lipid membranes and proteins, alcohols and anesthetics could have a multitude of targets, including both head and tail groups of lipids, membrane proteins, and/or the annular lipids of proteins (Franks and Lieb, 1987a;Franks and Lieb, 1987b). It is unlikely that any one of these targets can account for all of the alcohol and anesthetic effects in the CNS. More recent research has increased understanding of the interactions at each of these targets.

B. Glycine Receptors—Targets for Ethanol Action

Ligand-gated ion channels (LGICs) have received considerable attention as putative sites of ethanol action that cause its behavioral effects (Deitrich *et al.*, 1989;Harris, 1999;Mihic *et al.*, 1997;Ye *et al.*, 1998;Zhou and Lovinger, 1996;Cardoso *et al.*, 1999;Davies and Alkana,

2001;Molander and Söderpalm, 2005;Molander *et al.*, 2007;Rewal *et al.*, 2009). Research in this area has focused on investigating ethanol's effects on two large superfamilies of LGICs: 1) The cys-loop superfamily of LGICs (Ortells and Lunt, 1995;Karlin, 2002) whose members include nicotinic acetylcholine (nACh), 5-hydroxytryptamine₃ (5HT₃), γ -aminobutyric acid type-A (GABA_A) or type-C (GABA_C), and glycine receptors (GlyRs) (Mihic and Harris, 1996;Zhou and Lovinger, 1996;Grant, 1995;Cardoso *et al.*, 1999;Davies *et al.*, 2002;Davies *et al.*, 2004;Crawford *et al.*, 2007;Perkins *et al.*, 2008) and 2) The glutamate superfamily of LGICs including Nmethyl D-aspartate (NMDA), α -amino-3-hydroxyisoxazolepropionic acid (AMPA) and kainate receptors (Monaghan *et al.*, 1989;Sommer and Seeburg, 1992). These studies found that pharmacologically relevant concentrations of ethanol potentiate (i.e. allosterically increase or positively modulate) the action of GABA and glycine on GABA_ARs and GlyRs respectively. On the other hand, ethanol generally inhibits (i.e., allosterically decrease; negatively modulate) the actions of glutamate on its receptors (Criswell *et al.*, 1993;Deitrich *et al.*, 1989;Lin *et al.*, 1993;Franks and Lieb, 1994;Weight *et al.*, 1992;Mihic *et al.*, 1997;Ye *et al.*, 1998). Other cys-loop receptors such as GABA_C, nACh, and 5-HT₃Rs (Mihic and Harris, 1996;Zhou and Lovinger, 1996;Grant, 1995;Cardoso *et al.*, 1999;Davies *et al.*, 2006) as well as the ATP-gated purinergic (P2X) superfamily (Weight *et al.*, 1999;Xiong *et al.*, 1999;Davies *et al.*, 2002;Davies *et al.*, 2005;Asatryan *et al.*, 2008;Popova *et al.*, 2010) have also been shown to be sensitive to the effects of ethanol.

GlyRs are recognized as the primary mediators of neuronal inhibition in the spinal cord. In addition, they mediate inhibition in the brain stem and higher brain regions known to be sensitive to ethanol (Lynch, 2004;Molander and Söderpalm, 2005;Molander *et al.*, 2007). In addition to alcohol, GlyRs are modulated by a variety of other agents including zinc, picrotoxin, strychnine and ivermectin (Bloomenthal *et al.*, 1994;Laube *et al.*, 1995;Shan *et al.*, 2001;Webb and Lynch, 2007;Yang *et al.*, 2007;McCracken *et al.*, 2010). Functional GlyRs consist of five subunits which come together to form a single chloride ion channel. GlyR subunits cloned to date include four α subunits (1 to 4) and one β subunit, which provide for significant diversity (Lynch, 2004). These receptors undergo a developmental switch from $\alpha 2$ to $\alpha 1\beta$ by around postnatal day 20 in rat (Watanabe and Akagi, 1995). Adult GlyRs are found in a 2:3 $\alpha\beta$ stoichiometry (Webb and Lynch, 2007) however, most of the information obtained regarding the molecular structure and function of GlyRs, including ethanol studies, have used homomeric $\alpha 1$ GlyRs.

The significance of glycinergic neurons in defined neuronal circuits in the brain has not been sufficiently investigated. This is a matter of relevance with regards to functional interpretation of the roles of GlyRs in vivo because the distribution of GlyR subunits extends to areas in addition to glycinergic axon terminals (Araki *et al.*, 1988;Malosio *et al.*, 1991). To address this paucity of information regarding the precise innervation of GlyRs in neurons, a recent study by Zeilhofer and colleagues (Zeilhofer *et al.*, 2005) generated bacterial artificial chromosome (BAC) transgenic mice which expressed enhanced green fluorescent protein (EGFP). The EGFP in these mice was conditionally activated under the control of the neuronal glycine transporter 2 isoform (GlyT2) gene (Liu *et al.*, 1993), a reliable marker for glycinergic neurons in the CNS (Poyatos *et al.*, 1997;Ponce *et al.*, 1998). Using these transgenic animals, the authors were able to visualize the intensely fluorescent neurons expressing GlyT2-EGFP in great detail. This approach revealed an abundance of neurons exhibiting glycinergic signals (indicated by fluorescent innervation) in the hypothalamus, intralaminar nuclei of the thalamus and basal forebrain. In contrast, there was less abundance of positive fibers in the olfactory bulb, striatum, neocortex, hippocampus and amygdala. The authors reported a lack of GlyT2-EGFP-positive cell bodies in the forebrain. The authors also carried out double immunofluorescence staining between EGFP and GlyT1, the glycine transporter specific for glia, and reported that there was distinct separation between the two markers at almost all levels of the neuraxis (Zeilhofer *et al.*, 2005). Overall, these findings from Zeilhofer and colleagues (Zeilhofer *et al.*, 2005), point

out that the distribution of glycinergic axon terminals is less pervasive than glycine receptors. This finding has large implications for glycinergic signaling in the CNS, independent of the traditional, activity-dependent, synaptic release of glycine, especially when considering the actions of modulators such as alcohol and their behavioral effects mediated via GlyRs.

Building evidence consistently supports the notion that ethanol acting on GlyRs causes at least a subset of its behavioral effects and may be involved in modulating ethanol intake. GlyRs have been identified in key alcohol sensitive brain regions and play a critical role in modulating the excitability of neurons associated with sensory information and motor control of respiration (Rajendra *et al.*, 1997; Betz, 1991; Lynch, 2004; Webb and Lynch, 2007). These functions are significantly altered during ethanol intoxication. In addition, glycine and D-serine (a glycine pre-cursor) were shown to enhance ethanol-induced loss of righting reflex in mice (Williams *et al.*, 1995). The effect of glycine on the loss of righting reflex was blocked by strychnine, suggesting that glycine enhances the action of ethanol by acting on strychnine-sensitive GlyRs. This notion is consistent with other work showing that GlyRs mediate part of the immobility produced by anesthetics (Quinlan *et al.*, 2002; Zhang *et al.*, 2001; Zhang *et al.*, 2003). This conclusion is further supported by studies in which transgenic expression of S276Q mutant $\alpha 1$ GlyR subunits decreased sensitivity to ethanol-induced loss of righting reflex, motor incoordination (rotarod) and inhibition of strychnine-induced seizures (Findlay *et al.*, 2002). Moreover, recent studies using microdialysis infusions of glycine or strychnine into the nucleus accumbens suggest that GlyRs are important for regulating voluntary ethanol intake and may act as an entrance point into the brain reward system (Molander and Söderpalm, 2005; Molander *et al.*, 2007; Ericson *et al.*, 2009). In addition, studies suggest that Alko Alcohol/Non-Alcohol rats are differently disposed to ethanol consumption due to the arrangement or placement and/or compositions of their GlyRs (Jonsson *et al.*, 2009). This work found a strong positive correlation between $\alpha 1$ subunit expression in the nucleus accumbens and increased ethanol intake Alko Alcohol rats. Along the same lines, recent studies using $\alpha 1$ glycine null mutant mice suggests that the $\alpha 1$ subunit may be involved in ethanol consumption (Blednov and Harris, personal communication).

Electrophysiological studies of GlyRs further support a role for GlyRs in mediating the effects of ethanol on the brain. Behaviorally relevant concentrations of ethanol positively modulate GlyR function measured in synaptoneurosomes of whole-rat brain (Engblom and Åkerman, 1991), embryonic spinal neurons of mouse and chick (Celentano *et al.*, 1988; Aguayo and Pancetti, 1994; Tapia *et al.*, 1998; Ziskind-Conhaim *et al.*, 2003), freshly dissociated rat neurons (Ye *et al.*, 2001a; Ye *et al.*, 2001b; Ye *et al.*, 2002; Tao and Ye, 2002; Jiang and Ye, 2003; McCool *et al.*, 2003) and brain slice preparations (Eggers *et al.*, 2000; Eggers and Berger, 2004; Sebe *et al.*, 2003). In addition, ethanol reliably and robustly potentiates human recombinant $\alpha 1$ and $\alpha 2$ GlyRs measured electrophysiologically (Mascia *et al.*, 1996a; Mascia *et al.*, 1996b; Mihic *et al.*, 1997; Valenzuela *et al.*, 1998; Yamakura and Harris, 2000; Davies *et al.*, 2003; Davies *et al.*, 2004; Crawford *et al.*, 2007; Perkins *et al.*, 2008; Perkins *et al.*, 2009). Recent work by Mihic and colleagues examined the effects of ethanol on outside-out patches pulled from *Xenopus laevis* oocytes expressing $\alpha 1$ GlyRs (Welsh *et al.*, 2009). The authors reported that ethanol potentiates GlyR function by increasing burst durations and increasing the number of channel openings per burst. They reported minimal effects of alcohol on open and closed dwell times and likelihood. Similarly, the percentage of open time within bursts was not affected. Their kinetic modeling analyses suggest that ethanol increases burst durations by decreasing the rate of glycine unbinding (Welsh *et al.*, 2009).

Therefore, *in vitro*, pharmacological, molecular, electrophysiological and behavioral evidence indicates that ethanol-induced modulation of strychnine-sensitive GlyRs plays a key role in causing behavioral effects of ethanol and may influence ethanol intake through brain reward pathways.

C. Strategies for Identifying Sites and Mechanisms of Ethanol Action

Despite the advances in understanding the effects of ethanol *in vivo* and *in vitro* described above, the precise molecular sites and mechanisms of action for ethanol in ligand-gated ion channels in general, and in GlyRs specifically, are just now starting to become understood.

Part of the difficulty in identifying the sites of ethanol action, and in determining their roles in causing the effects of ethanol, reflects the physical-chemical nature of ethanol's mechanism of action. This physical-chemical, non-structure based, low affinity mechanism limits the ability to use standard pharmacological approaches to answer these questions. Findings over the last fifteen years suggesting that ethanol acts by "binding" to pockets (Franks and Lieb, 1994; Mihic *et al.*, 1997; Franks and Lieb, 1997; Ye *et al.*, 1998; Wick *et al.*, 1998; Mascia *et al.*, 2000; Crawford *et al.*, 2007) seem to have blurred the mechanistic distinction between intoxicant-anesthetics, such as ethanol, and other psychoactive drugs.

However, the primary determinant of intoxicant-anesthetic potency remains hydrophobicity, not molecular structure. Furthermore, the millimolar ethanol concentrations required for its biological actions are inconsistent with high affinity sites of action and suggest that ethanol acts simultaneously by the same mechanism on different types of initial sites (Deitrich *et al.*, 1989; Davies *et al.*, 2004). Hence, ethanol's physical-chemical mechanism of action is fundamentally different from the selective, high affinity binding mechanism that is known to initiate the behavioral effects of most psychoactive drugs (Dunn *et al.*, 1999). Therefore, we refer to the sites of ethanol action as "action sites" and "action pockets" to distinguish them from classical binding sites and to incorporate findings suggesting that there likely are multiple ethanol targets within one action pocket.

This atypical mechanism, and resultant lack of high affinity and pharmacological specificity, precludes the classical approach of using ethanol receptor antagonists to identify the sites and mechanisms of ethanol action and to establish cause-effect relationships (Lister and Nutt, 1987; Deitrich *et al.*, 1989; Little, 1991; Davies and Alkana, 2001). Presented next are several strategies which have been used to help fill this void.

(i) Molecular Biology—Techniques involving molecular manipulation, such as mutagenesis and chimeric strategies, have become a classical approach to study the effect of protein structure on function and response to drugs. In the GlyR, this strategy has been used with numerous assays, such as electrophysiology and biochemistry, to investigate the role that particular amino acid residues play in the effects of ethanol. Such molecular manipulations of receptor structure can be used alone or in combination with reagents that covalently bind to cysteine substituted residues and mimic the effect of "binding" small alcohol molecules. This technique is based on the premise that these covalently bound alcohol-like anesthetics [e.g., propanethiol or propyl methanethiosulfonate (PMTS)] will change the reversible effect of the agent to an irreversible effect only if the actions of the anesthetic result from acting at this site (Mascia *et al.*, 2000; Crawford *et al.*, 2007).

(ii) Pressure Antagonism of Ethanol—A second approach uses increased atmospheric pressure (pressure, hyperbaric exposure), in lieu of a traditional pharmacological antagonist, to help identify the sites and mechanisms of ethanol action. Studies show that exposure to 4–12 times normal atmospheric pressure (ATA) is a direct ethanol antagonist that blocks and reverses a broad spectrum of ethanol's acute and chronic behavioral effects (Alkana and Malcolm, 1981; Alkana and Malcolm, 1982a; Malcolm and Alkana, 1982; Alkana *et al.*, 1992; Nielsen *et al.*, 1987; Bejanian *et al.*, 1993; Davies *et al.*, 1994; Davies *et al.*, 1999; Davies and Alkana, 2001), as well as its effects at the biochemical (Davies and Alkana, 1998; Davies *et al.*, 1999; Davies and Alkana, 2001) and molecular (Davies *et al.*, 2003; Davies *et al.*, 2004; Perkins *et al.*, 2008) levels.

Pressure at this low level antagonizes ethanol without causing changes in behavior or baseline receptor function that called into question the specificity and utility of the higher pressures (>100 ATA) needed for pressure reversal of anesthesia (Hunter and Bennett, 1974; Halsey *et al.*, 1970; Brauer *et al.*, 1979; Miller, 1977; Wann and MacDonald, 1988). These studies at 12 ATA and lower demonstrate that pressure antagonism meets the key criteria for a direct mechanism (Syapin *et al.*, 1988; Bejanian *et al.*, 1993; Davies *et al.*, 1994; Syapin *et al.*, 1996; Davies *et al.*, 1999; Davies and Alkana, 2001; Malcolm and Alkana, 1982; Alkana and Malcolm, 1982b; Alkana and Malcolm, 1982a; Davies *et al.*, 2003) and selectivity (Alkana *et al.*, 1995; Davies and Alkana, 1998; Davies *et al.*, 1996; Davies and Alkana, 1998; Davies *et al.*, 2001; Davies *et al.*, 1999; Davies *et al.*, 2003), necessary for it to be used in a manner analogous to a traditional pharmacological antagonist to help identify initial molecular sites of ethanol action and to test cause-effect relationships (Davies and Alkana, 2001). From this perspective, the sites of pressure antagonism are the same as the sites of ethanol action. Hence, mutation of a site that alters the sensitivity of a receptor to ethanol should also alter its sensitivity to pressure antagonism, if the site is an initial site of ethanol action. Therefore, knowledge regarding the sites and mechanism(s) of pressure antagonism of ethanol in LGICs can identify new sites and mechanisms of ethanol action, which in turn can serve as new targets for drug development.

(iii) Molecular Modeling—The lack of a crystal structure greatly complicates traditional structure-function and or docking analyses of agonist and ethanol action in the GlyR. As a result, molecular modeling that incorporates evidence generated from the strategies outlined above is used to help define the sites of ethanol action in GlyRs, to suggest structure-function relationships and to identify possible sites of molecular interaction that mediate ethanol actions. Molecular models of GlyR and GABA_AR are typically built by combining multiple X-ray structures from unrelated organisms to create a suitable template for homology modeling (Trudell and Bertaccini 2004). These models have been validated by intermediate resolution cryo-electron microscopy structures of torpedo nAChR (PDB ID 2BG9) (Unwin 2005). This approach has already led to important advances.

Developing models that define the physical-chemical properties of the sites and mechanisms by which ethanol acts, that determine the properties that constitute its activation pockets, and that control ethanol sensitivity are key steps for developing agents to block, modulate and/or mimic the actions of ethanol at these sites.

Together, molecular, hyperbaric and modeling strategies provide complementary approaches for identifying, testing and depicting possible sites and mechanism(s) of ethanol action and antagonism, as well as sites that control ethanol sensitivity in a variety of receptors. The present review now focuses on advances in our knowledge produced by using these strategies over the last two decades to probe, identify and model the initial molecular sites and mechanisms of ethanol action in GlyRs.

2. REGION-SPECIFIC TARGETS AND MECHANISMS OF ETHANOL ACTION

The majority of studies that explored the sites and mechanisms of action of ethanol in GlyRs have focused on putative targets in the transmembrane (TM) and extracellular (EC) domains. However, a number of studies suggest that the intracellular domain may also be involved as a target and or mediator of the actions of ethanol in GlyRs. With this in mind, the following review primarily focuses on the TM and EC domains, but also includes a brief presentation regarding recent investigations focusing on the role of the intracellular domain.

A. INTRACELLULAR DOMAIN

Recent work found that multiple signal transduction pathways in the intracellular domain of GlyRs were modulated by pharmacologically relevant concentrations of ethanol (Morrow *et al.*, 2004; Ron and Jurd, 2005). Additional studies suggested discrete molecular determinants of ethanol sensitivity within the large intracellular loop of this receptor. This later work focused on intracellular signaling in the GlyR initiated by PKA, PKC and G $\beta\gamma$ dimmers (Legendre, 2001; Lynch, 2004; Harvey *et al.*, 2004). Further work revealed that α_1 GlyRs are modulated by G proteins through G $\beta\gamma$ heterodimers (Yevenes *et al.*, 2006). Interestingly, recent studies have shown that the degree of GlyR-G $\beta\gamma$ functional interaction is critical for ethanol-induced potentiation on the glycine-activated current (Yevenes *et al.*, 2008). Subsequent related work found that ethanol effects on the GlyR could be blocked by a peptide that interferes with G $\beta\gamma$ binding (Guzman *et al.*, 2009). Taken together, this work suggests that intracellular signaling elements, such as G proteins play a role in controlling ethanol sensitivity and possibly initiating ethanol action in GlyRs.

B. TRANSMEMBRANE DOMAIN

Most early studies investigating the initial targets for ethanol action in GlyRs focused on the TM region, which comprises the channel pore. Initial investigations into the molecular targets for ethanol within cys-loop receptors utilized two receptors: α_1 GlyRs and ρ_1 GABA_ARs (Mihic *et al.*, 1997). Both receptors form functional homomers and have qualitatively different responses to ethanol (α_1 GlyRs are potentiated by ethanol and ρ_1 GABA_ARs are inhibited by ethanol). The authors developed chimeras of these receptors and used these chimeras as a tool to begin to search for potential important regions of the receptor for the action of ethanol. This was accomplished by testing various mutant receptors containing various segments of the protein and determining which portion of the receptor could be spliced into the corresponding location of the other receptor to change the ethanol response. This study identified a 45 amino acid residue region containing TM2 and TM3 that appeared to be both necessary and sufficient for ethanol modulation of receptor function. Sequence alignment comparison of α_1 GlyRs and ρ_1 GABA_ARs revealed twelve differences at homologous residues within this 45 amino acid residue region. Replacing the amino acids at these positions in α_1 GlyRs with the respective residues of the ρ_1 GABA_AR identified specific TM2 and TM3 residues (S267 and A288 in the α_1 GlyR) that are critical for allosteric modulation of these receptors by alcohols and volatile anesthetics.

Subsequent studies found that systematic replacement of the serine at position 267 with the other nineteen amino acids altered ethanol potentiation of GlyR function (Ye *et al.*, 1998). Moreover, this work revealed an inverse correlation between molecular volume of the amino acid substitution and the effect of ethanol, with a crossover from ethanol potentiation to inhibition beginning at residues equal to or larger in volume than isoleucine. Potentiation by alcohols and anesthetics was also altered by mutations at I229 in TM1, A288 in TM3, and W407 and Y410 in TM4 (Greenblatt and Meng, 1999; Jenkins *et al.*, 2001; Yamakura *et al.*, 1999; Lobo *et al.*, 2004a; Lobo *et al.*, 2006). Together, these results demonstrate that the amino acid residues in TM domains can play a crucial role in determining the functional consequences of allosteric modulation of the GlyR by alcohols and anesthetics.

Further investigations used the n-chain alcohol cutoff to test the possibility that ethanol has a finite site of action in the TM domain. The cutoff refers to the failure to increase potency as a function of increasing the n-chain alcohol length and is thought to occur when the molecular volume of the alcohol exceeds the finite volume of a putative alcohol pocket (Wick *et al.*, 1998; Pringle *et al.*, 1981; Alifimoff *et al.*, 1989). Prior work had established that different ligand-gated ion channels exhibit different cutoff points (the carbon chain length for GlyRs and GABA_ARs = 10–12; GluRs, AMPARs and KARs = 7; ρ_1 GABA_ARs = 7 and P2XRs =

3) (Dildy-Mayfield *et al.*, 1996; Mascia *et al.*, 1996a; Mihic and Harris, 1996; Li *et al.*, 1994), supporting the existence of alcohol “binding” pockets of variable size on these membrane proteins (Wick *et al.*, 1998). Therefore, to test the hypothesis that the TM domains of $\alpha 1$ GlyRs and $\rho 1$ GABA_ARs could form an alcohol binding pocket, mutations were performed on TM2 residues and the alcohol cutoff was observed. Substituting larger residues into position 267 of the $\alpha 1$ GlyR significantly decreased the alcohol cutoff, whereas mutation of the homologous position in the $\rho 1$ GABA_AR to smaller residues increased the cutoff. These results support the existence of alcohol action/“binding” pockets within each of these receptors and suggest that the amino acid residues present within the TM domains can control the size of the alcohol action cavity.

Subsequent experiments tested the hypothesis that amino acids S267 in TM2 and A288 in TM3 in the $\alpha 1$ GlyR represent critical action/“binding” sites for alcohols and anesthetics by using cysteine mutagenesis and anesthetic-like thiol or methanethiosulfonate (MTS) reagents (Mascia *et al.*, 2000). The authors suggested that the creation of a disulfide bond following covalent binding at a single site would change the reversible potentiating effect of the agent to an irreversible effect if the actions of the alcohol or anesthetic result from binding at this site. Consistent with their hypothesis, propanethiol or propyl methanethiosulfonate (PMTS) exposure to $\alpha 1$ S267C GlyRs caused the expected change. Furthermore, PMTS binding to position 267 abolished the potentiating effects of enflurane, isoflurane and octanol suggesting competition for a single binding site among PMTS and alcohols and anesthetics. When in the resting state of the receptor, neither propanethiol nor PMTS were able to irreversibly enhance the function of the A288C TM3 mutant or prevent action of isoflurane on these receptors. In addition, ethanol, in the absence of agonist, causes a conformational change in the receptor that can change accessibility of TM residues (Jung *et al.*, 2005; Jung and Harris, 2006). Finally, PMTS occupancy of a single anesthetic binding pocket within the GlyR pentamer is sufficient to enhance receptor function (Roberts *et al.*, 2005). Taken together, these studies support the notion that TM position 267 is capable of initiating the actions of ethanol.

Recent structure-function studies of the GlyR using MTS reagents identified other positions that may be involved in producing the actions of ethanol and showed the orientation of these amino acids and their importance in channel function. These results have been reported in several original articles and a review article (Lobo *et al.*, 2004a; Lobo *et al.*, 2004b; Lobo and Harris, 2005; Lobo *et al.*, 2006; Lobo *et al.*, 2008). Amino acids in TM1 (I229C), TM2 (M263C, S267C, S270C), and TM3 (A288C) were identified as being accessible to PMTS and it was proposed that these residues define a cavity (Lobo *et al.*, 2004a). Furthermore, larger MTS reagents were used to determine if the volume of this cavity is different in the absence (resting state) and presence of glycine, and concluded that channel gating changes the conformation of this region, providing a mechanism by which occupation of this cavity by alcohols might alter channel function (Lobo *et al.*, 2004a). Subsequent work tested a series of cysteine mutants in TM4 and found that W407C, I409C, Y410C, and K411C showed evidence of reaction with propyl MTS, and mutations at these sites eliminated actions of ethanol on the GlyR (Lobo *et al.*, 2006).

These observations raised the critical question of whether these amino acids in TM 1, 2, 3 and 4 all face a common space or cavity. Because there is no detailed (e.g., X-ray diffraction) structural information for the GlyR, cysteines were introduced into two of these positions to determine if they were sufficiently close in space to allow crosslinking. These cross linking studies provided evidence for cross-linking of the TM2,3 regions (S267C-A288C) and the TM1,3 regions (I229C-A288C) (Lobo *et al.*, 2004b; Lobo *et al.*, 2008).

Taken together, these results are consistent with the notion that LGICs are dynamic proteins during alcohol modulation and channel gating. Collectively, the findings support the hypothesis

that the TM domains contribute to an alcohol action/"binding" pocket and that specific residues play a role in causing alcohol modulation of GlyR function.

C. EXTRACELLULAR DOMAIN

The extracellular domain of GlyRs and GABA_ARs is recognized as the site of agonist binding and receptor activation (Kash *et al.*, 2003; Kash *et al.*, 2004b; Chakrapani *et al.*, 2004; Crawford *et al.*, 2008). The EC domain also contains the sites for binding of allosteric modulators like Zn²⁺ and benzodiazepines (Sigel and Buhr, 1997; Mhatre and Ticku, 1989; Kucken *et al.*, 2000; Wieland *et al.*, 1992; Barberis *et al.*, 2002; Bloomenthal *et al.*, 1994). A recent study suggests that the synergistic interaction between zinc ions and ethanol might involve the extracellular domain (McCracken *et al.*, 2010), as this region is thought to contain the most likely high- and intermediate affinity binding sites for zinc (Bloomenthal *et al.*, 1994; Laube *et al.*, 1995). Binding to the benzodiazepine site in GABA_ARs is known to cause both positive and negative (inverse) agonist modulation. Recent studies suggest that the benzodiazepine inverse agonist Ro15-4513 antagonizes alcohol by binding to a non-benzodiazepine binding site in the EC domain (Mehta and Ticku, 1989; Wallner *et al.*, 2006). Moreover, hyperbaric studies suggest that pressure antagonizes ethanol-induced chloride uptake by uncoupling a benzodiazepinelike allosteric modulation of the receptor (Davies and Alkana, 2003). Taken together, these findings provide several lines of indirect evidence suggesting that alcohol may have sites of allosteric action in the EC domain of GlyRs and GABA_ARs.

Recent studies using recombinant expression systems provide more direct evidence that ethanol also acts on the extracellular domain in GlyRs. The initial suggestion by Harris and colleagues came from recombinant studies of GlyRs which demonstrated that α 1 GlyRs were more sensitive to ethanol than α 2 GlyRs (Mascia *et al.*, 1996b). These studies found that an alanine to serine exchange at position 52 (A52S), located at the beginning of extracellular domain Loop 2 (Brejc *et al.*, 2001) and one of the residues that differs between α 1 and α 2 GlyRs, decreased the ethanol sensitivity of the α 1 GlyR to the point where the ethanol response resembled that of the α 2 GlyR. Overall, the findings implicated residues in the extracellular domain, particularly position 52, as possible sites of ethanol action and/or modulation.

Subsequent studies by Davies et al used a novel ethanol antagonist, increased atmospheric pressure, to probe the role of extracellular domain position 52 in ethanol action. This work found that pressure antagonized ethanol in wildtype (WT) α 1GlyRs, but did not antagonize ethanol in mutant A52S GlyRs (Davies *et al.*, 2003; Davies *et al.*, 2004). Moreover, they found that pressure did not antagonize ethanol in α 2 GlyRs, which have a threonine at the homologous position (T59) to position 52 in α 1GlyRs. Interestingly, the converse experiment, substituting alanine for threonine at position 59 in α 2 GlyRs, increased ethanol sensitivity and conferred sensitivity to pressure antagonism of ethanol, thereby making α 2GlyRs more α 1-like (Perkins *et al.*, 2008). This ability of a single point mutation to alter sensitivity to both ethanol and pressure antagonism provided key evidence that extracellular domain position 52 is a site of ethanol action.

Studies by Crawford et al tested the hypothesis that position 52 is an initial target for ethanol action in α 1GlyRs and began to study possible relationships between position 52 in the EC domain and position 267 in the TM domain (Crawford *et al.*, 2007). They accomplished this by investigating the effects of cysteine substitutions at positions 52 and position 267 on the responses of α 1GlyRs to n-alcohols and PMTS. Based on the earlier studies in the TM domain described above (Mascia *et al.*, 2000; Borghese *et al.*, 2003), the authors predicted that PMTS would cause irreversible potentiation in A52C GlyRs when bound to the substituted cysteine at the site, if the site is capable of causing alcohol-like receptor modulation. As predicted, PMTS caused irreversible potentiation in A52C, but not WT GlyRs (Fig. 1A). Taken with prior

studies at positions 267, these findings support the hypothesis that both positions 52 and 267 are initial sites of ethanol action in $\alpha 1$ GlyRs.

Further studies used sequential application of PMTS followed by ethanol in cysteine-substituted $\alpha 1$ GlyRs to isolate the effects of ethanol on positions 52 and/or 267 (Crawford *et al.*, 2007). Crawford and colleagues reasoned that PMTS would bind to and saturate the cysteine-substituted residues, and would thus prevent further modulation through these bound sites. This is analogous to a pharmacological antagonist binding to and blocking the action of the agonist on the site. The results, summarized in Fig 1, show that ethanol reversibly potentiated (positively modulated) $\alpha 1$ GlyRs when position 52 is occupied by PMTS (Fig 1B), that ethanol negatively modulated $\alpha 1$ GlyR when position 267 is occupied by PMTS (Fig 1C) and that ethanol produced a small degree of negative modulation when positions 52 and 267 are both occupied by PMTS (Fig 1D). These findings led the authors to conclude that: 1) Positions 52 and 267 are sites of ethanol action that are capable of causing ethanol effects when the other is blocked; 2) Ethanol causes qualitatively different effects depending on its site of action in the GlyR--ethanol acting on position 52 produces negative modulation, whereas ethanol produces positive modulation when it acts on position 267 and 3) Positions 52 and 267 are not the only targets for ethanol in $\alpha 1$ GlyRs based on the residual effect of ethanol in the double mutant. Collectively, these findings provided evidence that ethanol's effect on $\alpha 1$ GlyRs represents the summation of its actions on different targets in the EC and TM domains.

Studies from different laboratories come together to provide insight into whether the sites of action for ethanol in the EC and TM domains of $\alpha 1$ GlyRs are part of the same alcohol action pocket. As described above, Harris and colleagues found that WT $\alpha 1$ GlyRs have a cutoff between decanol and dodecanol and that exposing S267C mutant $\alpha 1$ GlyRs, but not WT GlyRs, to PMTS reduced the cutoff to below octanol (Mascia *et al.*, 1996a; Mascia *et al.*, 2000). Subsequent studies by Crawford *et al.* showed that exposing A52C $\alpha 1$ GlyRs to PMTS reduced the cutoff to between octanol and decanol (Crawford *et al.*, 2007). Whereas, exposing the double mutant (A52C-S267C) GlyRs to PMTS appeared to reduce the cutoff to below hexanol. Since PMTS changed the cutoff in both A52C and S267C GlyRs, these findings led Crawford *et al.* to conclude that neither position 52 nor 267 belong to a separate pocket that can independently account for the WT cutoff. Therefore, these findings supported the notion that position 52 in Loop 2 of the EC domain and position 267 in the TM domain are part of the same alcohol action pocket (Crawford *et al.*, 2007) and led to a new molecular model of an alcohol pocket spanning from position 52 in the extracellular domain to position 267 in the TM domain (See Fig 3 in the next section on molecular models).

More recent studies investigated the relationships between the physical-chemical properties of the residue at position 52 in $\alpha 1$ GlyRs and the impact of these properties on the sensitivity of the receptor to ethanol. This work was based, in part, on the A52S mutant GlyRs studies described above that suggested a role for polarity of the residues at position 52 in $\alpha 1$ GlyRs, and the homologous position 59 in $\alpha 2$ GlyRs, in determining the sensitivity of the respective receptors to ethanol (Mascia *et al.*, 1996b; Davies *et al.*, 2004). To accomplish this, Perkins *et al.* systematically varied the molecular weight, volume, polarity and hydrophobicity of the residue at position 52 and determined the effect of these changes on ethanol sensitivity (Perkins *et al.*, 2008). They found that increasing the polarity of the residue at position 52 significantly reduced sensitivity to ethanol compared to WT. Correlation analyses indicated that polarity explained approximately 88% of the variability in the sensitivity of $\alpha 1$ GlyRs to ethanol, while hydrophobicity accounted for approximately 69% of the variability. This correlation was independent of changes in the glycine EC₅₀. Molecular volume and weight of the residues at position 52 did not appear to affect the sensitivity of $\alpha 1$ GlyRs to ethanol.

The authors pointed out that their findings with polarity at position 52 in the extracellular domain (Perkins *et al.*, 2008) differed from previous studies by others in the TM domain (Ye *et al.*, 1998). The latter showed a role for molecular volume, but not polarity, of the residue at position 267 in controlling ethanol sensitivity in $\alpha 1$ GlyRs. Based on these differences, Perkins and colleagues concluded that different physical-chemical properties might influence ethanol action, respectively, in the EC and TM domains.

The findings described above demonstrate that the physical-chemical properties of the residue at position 52 can influence the sensitivity of $\alpha 1$ GlyRs to ethanol. Recent studies brought attention to other positions in Loop 2 (Crawford *et al.*, 2008). Cysteine point mutations at positions 50–60 (Loop 2) in $\alpha 1$ GlyRs can significantly alter glycine sensitivity. Subsequent work reported that cysteine substitutions at several positions in Loop 2 changed the receptor's sensitivity to ethanol (Crawford *et al.*, 2006). Moreover, application of PMTS to these cysteine mutants produced irreversible modulation in some of the mutant receptors (Crawford *et al.*, 2006). These findings suggest that other residues in Loop 2 also play a role in initiating and/or modulating the actions of ethanol on $\alpha 1$ GlyRs.

Collectively, the demonstrations that the physical-chemical properties of the amino acid at position 52 alter the sensitivity of $\alpha 1$ GlyRs to ethanol and that there are multiple positions in Loop 2 that can also affect ethanol sensitivity suggested that the structure of Loop 2 may play an important role in determining the ethanol sensitivity of $\alpha 1$ GlyRs, and other receptors (Perkins *et al.*, 2008). To test this possibility, the authors identified WT receptors in GlyRs and GABA_ARs that had marked differences in ethanol sensitivity and differed in their Loop 2 structures. Based on prior studies, delta (δ) subunit-containing extrasynaptic GABA_ARs were chosen as the highly ethanol sensitive representative. This choice was based on prior work, although somewhat controversial (Borghese *et al.*, 2006; Borghese and Harris, 2007) that delta (δ) subunit-containing extrasynaptic GABA_ARs are sensitive to low ethanol concentrations (3–30 mM) in a variety of preparations (Wallner *et al.*, 2003; Wei *et al.*, 2004; Liang *et al.*, 2006; Hancher *et al.*, 2005; Hancher *et al.*, 2006; Wallner *et al.*, 2006; Fleming *et al.*, 2007; Glykys *et al.*, 2007; Mody *et al.*, 2007; Santhakumar *et al.*, 2007). To represent receptors that had less sensitivity to ethanol in recombinant preparations, the authors selected gamma (γ) subunit-containing synaptic GABA_ARs which are insensitive to low (1–30) ethanol concentrations (White *et al.*, 1990; Weiner *et al.*, 1997) and GlyRs which are also not sensitive to ethanol concentrations below 10 mM (Davies *et al.*, 2003; Mihic *et al.*, 1997; Perkins *et al.*, 2008). A comparison of the Loop 2 sequences of GABA_AR δ , GABA_AR γ and GlyR $\alpha 1$ subunits revealed differences in the residues at homologous Loop 2 positions in these receptors that could underlie their differences in ethanol sensitivity. Based on this information, the authors predicted that mutating Loop 2 of $\alpha 1$ GlyRs to the homologous sequence in Loop 2 in δ GABA_ARs, would increase the ethanol sensitivity of the mutant $\alpha 1$ GlyRs, if the Loop 2 δ sequence played a key role in determining ethanol sensitivity in these receptors (Perkins *et al.*, 2009).

Perkins and colleagues tested this notion by developing mutant $\alpha 1$ GlyRs, in which multiple mutations were performed that replaced the Loop 2 region of the GlyR $\alpha 1$ subunit (positions 50–59) with the homologous residues from the GABA_AR δ subunit (positions 43–52) (Perkins *et al.*, 2009). This mutant was designated as $\alpha 1$ δ L2GlyR. In addition, mutations were performed that replaced the Loop 2 region of the GlyR $\alpha 1$ subunit (positions 50–59) with the homologous residues from the GABA_AR γ subunit (positions 64–73). This mutant was designated as $\alpha 1$ γ L2GlyR. As predicted, substituting the δ L2 in $\alpha 1$ GlyRs reduced the threshold for ethanol response from 30 mM in WT to 1 mM or less in the mutant. In addition, the mutation significantly increased the degree of ethanol potentiation at the low concentrations tested (1–30 mM). Substituting the γ L2 sequence did not affect the threshold or magnitude of ethanol response in this mutant GlyR. Parallel studies found that substituting Loop 2 of the γ subunit

of $\alpha 1\beta 2\gamma 2$ GABA_ARs with δ L2 markedly decreased the ethanol threshold and increased ethanol response of these GABA_ARs without altering receptor sensitivity to GABA or changing other aspects of receptor function (Perkins *et al.*, 2009).

These findings with δ L2 mutations in GlyR and GABA_AR support the hypothesis that the structure of Loop 2 can markedly change the threshold and magnitude of the response to ethanol in GlyRs. The results also demonstrate that this phenomenon extends to at least one other cysteine-loop receptor (GABA_AR). This work provided the basis for developing new molecular models of the GlyR threaded on the X-ray structure of the prokaryotic *Gloeobacter violaceus* pentameric ligand-gated ion channel homologue (GLIC) (Bocquet *et al.*, 2007; Bocquet *et al.*, 2009) presented in the next section of this review (Fig 3).

Collectively, these findings establish that Loop 2 in the EC domain contains multiple sites of action for ethanol and that the effects of ethanol on GlyR function reflect the summation of its actions on sites in the intracellular, EC and TM domains. Moreover, these findings also establish that the structure of Loop 2 in the extracellular domain can have a marked influence on ethanol sensitivity.

3. DISCUSSION: MOLECULAR MODELS AND MECHANISMS OF ETHANOL ACTION IN GLYCINE RECEPTORS

The lack of a crystal structure precludes traditional structure-function and/or docking analyses of agonist and ethanol action in the GlyR. This disadvantage has forced the use of molecular modeling, which incorporates experimentally obtained data to help define the sites of and possible mechanisms for ethanol action in GlyRs.

Although this review focuses on the molecular targets for ethanol actions in GlyRs and uses results from recent models to explain possible mechanisms, it would not be complete without mention of the lipid component of nerve membranes as a site of action. The influence of the Meyer-Overton correlation of solubility in olive oil with potency of alcohols and anesthetics (Meyer, 1899; Overton, 1901) suggested the possible influence of alcohol on membrane structure. Studies have previously investigated the effect of alcohol (Mastrangelo *et al.*, 1979) and inhalational anesthetics (Trudell *et al.*, 1973b) on membrane fluidity and protein-lipid interactions (Trudell, 1977; Huang *et al.*, 1995). There is clear evidence that membrane properties modulate the function of integral membrane proteins (Baldwin and Hubbell, 1985; Lee, 2009). However, as recently reviewed (Hemmings, Jr. *et al.*, 2005; Lobo and Harris, 2005; Harris *et al.*, 2008), the ability of single site-directed mutations in LGICs to modulate potentiation by alcohol directs our focus to these protein sites.

A. Molecular Models of Ethanol's Sites of Action

Molecular modeling in GlyRs has come a long way and yet, a cohesive explanation for the mechanisms of ethanol action in GlyRs is far from clear. The new nAChR structure (PDB ID 2BG9) (Unwin, 2005) has been used extensively as a template for molecular modeling of GlyR and GABA_AR (Bertaccini *et al.*, 2005; Arias *et al.*, 2006; Lobo *et al.*, 2006; Bertaccini *et al.*, 2007a; Bertaccini *et al.*, 2007b; Crawford *et al.*, 2007; Lobo *et al.*, 2008; Trudell *et al.*, 2008). However, questions remain about the exact position of many amino acid residues in the template as well as the alignment of the template with GlyR and GABA_AR (Bertaccini and Trudell, 2002; Ernst *et al.*, 2005). Many of these issues were resolved in a series of modeling and mutagenesis experiments (Jung *et al.*, 2005; Lobo and Harris, 2005; Jung and Harris, 2006; Lobo *et al.*, 2006; Crawford *et al.*, 2007; Lobo *et al.*, 2008; Crawford *et al.*, 2008; Harris *et al.*, 2008; Perkins *et al.*, 2008; Trudell *et al.*, 2008; Perkins *et al.*, 2009).

These initial models showed that the coupling between the extracellular (ligand binding) and TM domains is mediated by a single peptide link plus van der Waals and electrostatic interactions between Loops 2 and 7 from the ligand-binding domain and the TM2-3 loop in the TM domain (Kash *et al.*, 2003; Kash *et al.*, 2004a). Nevertheless, this coupling is sufficiently strong to convey the energy of agonist binding over 45 Å to the gate in the ion pore (Corringer *et al.*, 2000; Karlin, 2002; Unwin, 2005; Purohit and Auerbach, 2007). There is reduced electron density at the ligand-binding and transmembrane interface in the cryo-electron micrographs of nAChR (Unwin, 2005). This low packing density is substantiated by experiments because this region is accessible to MTS reagents, including the TM2-TM3 Loop (Lynch *et al.*, 2001; Bera *et al.*, 2002; Goren *et al.*, 2004) and a putative water-filled cavity that includes the extracellular one third of each subunit (Williams and Akabas, 1999; Mascia *et al.*, 2000). Reactivity of site-directed cysteine substitutions with alkyl-MTS reagents in the interfacial region implies that the cysteine thiol groups are ionized and, therefore, that the region is solvated by water (Bera *et al.*, 2002).

Recent findings in the extracellular and TM domains led to a new molecular model of a potential alcohol action pocket in GlyRs (Crawford *et al.*, 2007) (Fig 2). These findings, described in the prior section, indicate that position 52 in the extracellular domain and position 267 in the TM domain are both capable of initiating the actions of ethanol and that they are both part of the same alcohol action pocket. The model revealed a cavity that extends approximately 28 Å from the C α atoms of A52 to S267 that could function as this alcohol action pocket. The proposed pocket could hold several ethanol molecules and fewer larger alcohols. The estimated distance between position 52 and 267 precludes action by one ethanol molecule at both sites simultaneously, and suggests that at low concentrations, ethanol can enter and move in the pocket without touching one of these action sites. As the concentration of ethanol increases, the probability that ethanol molecule(s) will be acting on one or more of these sites at a given moment increases. The net response to ethanol on receptor function will represent the summation of the actions of ethanol on these targets. This model gives the first depiction of an alcohol action pocket in the EC-TM interface. Further study is necessary to investigate the model, to map the role of other EC domain residues (within and outside of Loop 2) in the actions of ethanol, to discover if other alcohol action pockets exist, and to investigate whether the present findings generalize to other LGICs.

B. Molecular Mechanisms of Ethanol Action

The physical chemical mechanisms by which the actions of ethanol on its targets are transduced into changes in the receptor's response are not well understood. Recent advances in molecular models have begun to provide new insights.

Physical evidence to date suggests alcohol binding to be weak and involve rapid on- and off-rates. The high K_d of alcohol (approximately 100 mM) requires that the binding energies to sites of action are small. As a result, it is not expected that the binding event could cause an "induced fit" in a protein site. Rather, it is likely that these molecules bind adventitiously to pre-existing cavities or sites (Cheng *et al.*, 2008; Harris *et al.*, 2008). These small molecules could affect function if there is equilibrium between the conformations of resting channels and those in the open/desensitized state (Schwartz and Hubbell, 2008). Binding of small ligands could change that equilibrium. One could further hypothesize that the ligand binds to most or all conformations of the receptor with varying affinities, but that the shapes or volumes of the binding sites differ between conformations. In that case, a given ligand could fit a site in one conformation better than another and provide more stabilization to that conformation.

This suggestion leads to the possibility that, on a molecular level, a site-directed mutation could switch which conformation is most stabilized by a particular ligand. In fact, there are now several examples in which single mutations change the relative potency of alcohols and

inhalational anesthetics (Greenblatt and Meng, 1999; Greenblatt and Meng, 2001; Jenkins *et al.*, 2001; Raines *et al.*, 2001; Scheller and Forman, 2001; Wenningmann *et al.*, 2001; Davies *et al.*, 2004; Woodward *et al.*, 2004; Perkins *et al.*, 2008). Models of alcohol binding sites include internal cavities in proteins (Rashin *et al.*, 1986; Eriksson *et al.*, 1992; Quillin *et al.*, 2000). Adventitious binding of solvents to internal cavities in mutants of T4 lysozyme (Blaber *et al.*, 1995; Morton *et al.*, 1995; Morton and Matthews, 1995) also provides a framework for this model.

The possibility that mutations can alter the sensitivity to ethanol is strongly supported by the recent demonstration by Perkins and colleagues (Perkins *et al.*, 2009) that changes in the structure of Loop 2 can produce 10 fold increases in the sensitivity of GlyR and GABA_AR described in the prior section. This finding led to new models of the GlyR that suggest possible physical chemical mechanisms by which ethanol can modulate the receptor's response to agonist (Fig 3) (Perkins *et al.*, 2009). As described by the authors, these models were developed using the high resolution template provided by the recent publication of an X-ray crystal structure of a prokaryotic ligand-gated ion channel homologue from *Gloeobacter violaceus* (GLIC, PDB ID 3EAM) (Bocquet *et al.*, 2009; Hilf and Dutzler, 2009). The new GlyR models were built by threading the primary sequence WT and Loop 2 mutant GlyRs onto the GLIC template using the Modeler module of Discovery Studio (DS 2.1; Accelrys, Inc, San Diego, CA) as previously described (Bertaccini *et al.*, 2005; Crawford *et al.*, 2007; Crawford *et al.*, 2008; Perkins *et al.*, 2009).

The authors propose that the resultant models offer a mechanistic explanation for ethanol's effects on the GlyR based on changes in Loop 2 structure (Perkins *et al.*, 2009). This mechanism is revealed by juxtaposing the models derived from threading the WT versus the δ L2 GlyR sequences onto GLIC. The change in conformation as a result of the δ L2 substitution in mutant GlyRs alters the manner in which Arg218 (pre-TM1) interacts with Glu53 (Loop 2) with a much different form of salt bridge. The delocalized charge of the three partially positive nitrogen atoms (N-H⁺ groups) at the guanidinium end of the Arg sidechain allows it to form a salt bridge with the Glu carboxyl group either straight-on (the longest net distance) or at the side of the arginine side chain (shorter net distance and not linear). The result of the δ L2 mutation is to form the more distorted side-on salt bridge in the modeling. This change causes a small distortion of the pre-TM1 segment compared to the WT GlyR. Moreover, the δ L2 mutant GlyR has an Asp residue at position 57 in place of the Glu found in WT. As expected, the substitution of Asp57 with Glu results in a shift of the salt bridge with Lys104 to compensate for the increased length of the Glu side chain. The side chain of Lys276 extends out from the TM2-3 linker to make contact with the conserved Glu53 in Loop 2, forming an inter-subunit salt bridge. It is possible that the solvation/desolvation of this salt bridge is important for the structural rearrangements that accompany the gating transition (Honig and Hubbell W.L., 1984). Moreover, the salt bridge between Arg218 and Glu53 has a different conformation in the δ L2 mutant GlyR. The altered length of this salt bridge may contribute to the differences in sensitivity to glycine and ethanol of the δ L2 mutant GlyR. The partial negative charges on Glu53, at the tip of the beta turn in Loop 2, are shared between Arg218 and Lys276. The salt bridge described above had been previously modeled within a single subunit (Kash *et al.*, 2003; Kash *et al.*, 2004a; Kash *et al.*, 2004b). In this most recent model of the GlyR by Perkins and coworkers (Perkins *et al.*, 2009), the salt bridge was flexible enough to extend across the inter-subunit space to contact the adjacent subunit.

Perkins and colleagues proposed that these complicated electrostatic and steric interactions might be especially sensitive to, and altered by, the presence of ethanol molecules in the adjacent cavity. These findings exploring the role of Loop 2 and the δ GABA sequence, exemplify how increasing our knowledge regarding the structures that can modulate ethanol

sensitivity can increase our understanding of the targets for ethanol and structure-function relationships.

The authors further point out that it is unlikely that these are just static changes. Rather, they would change the ensemble of conformations that may occur during gating, and may be affected by the presence of alcohol molecules, which could alter ethanol sensitivity. If valid, this suggests that these dynamic movements are involved in causing and/or transducing the action of ethanol in Loop 2. Despite the low homology between Loop 2 residues in $\alpha 1$ GlyR and δ GABA_AR, the global structure of the beta turn is conserved in the chimera, illustrating the importance of structural homology across the cys-loop superfamily. This suggests that insights provided by the current model may generalize to GABA_ARs and other members of the superfamily.

Taken together, the development of models that define the physical-chemical properties of the sites and mechanisms by which ethanol acts, that determine the properties that constitute its activation pockets, and that control ethanol sensitivity are key steps for developing agents to block, modulate and/or mimic the actions of ethanol at these sites.

The present review incorporates evidence regarding the actions of ethanol on GlyRs from a global perspective with its inclusion of findings from the intracellular, transmembrane and extracellular regions. Overall, the material reviewed summarizes advances that have increased our knowledge regarding the structures that initiate, transduce, modulate and antagonize the actions of ethanol on GlyRs and, likely, other LGICs. These findings have changed the thinking and models about the sites and mechanisms of ethanol action and contribute to the long-term goal of the field, which is to identify novel targets for medications development to prevent and treat alcohol abuse and dependence.

ABBREVIATIONS

5HT3R	5-hydroxytryptamine ₃ receptor
(ATA)	atmospheric pressure
(EGFP)	enhanced green fluorescent protein
(GABA _A R)	Gamma-aminobutyric acid type-A receptor
(GLIC)	Gloeobacter violaceus pentameric ligand-gated ion channel homologue
(GlyR)	glycine receptor
(GlyT2)	glycine transporter 2
(LGIC)	ligand-gated ion channel
(MTS)	methanethiosulfonate
(nAChR)	nicotinic acetylcholine receptor
(PMTS)	propyl methanethiosulfonate
(TM)	transmembrane
(WT)	wild type

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Fig. 1. PMTS binding to cysteines substituted at position 52 and/or 267 reveals position-specific negative and positive modulation by ethanol in $\alpha 1$ GlyRs

Mean $\pm$ SEM percent control glycine response for (A.) WT and (B. – D.) mutant $\alpha 1$ GlyRs ($n = 4 - 9$). Shaded boxes indicate GlyR responses in the presence of PMTS (grey) or 100 mM ethanol (yellow). (* $p < 0.05$ vs. control glycine EC_{10} response, † $p < 0.05$ versus residual effect of PMTS). Fig. from Crawford et al. 2007 (Crawford *et al.*, 2007).

Fig. 2. Zoom view of a molecular model of a α 1GlyR subunit with the alcohol pocket highlighted
The backbone atoms of one α 1GlyR subunit are shown as a yellow ribbon. The largest cavity found by the Binding Site Analysis module of Insight 2005L is shown in red. The C α atoms of A52 and S267 are separated by approximately 28Å. The interconnected sections of the pocket are large enough to accommodate ethanol and allow its passage between these regions. The ability of the A52C-S267C double mutant to gate currents and bind PMTS is consistent with there being enough distance between positions 52 and 267 to prevent spontaneous disulfide linkage between the cysteine substitutions. *Figure modified from Crawford et al. 2007 (Crawford et al., 2007).*

Fig. 3.
(A) Loop 2 in a single GlyR WT subunit. Residues interacting with Loop 2 are rendered as colored stick models: Lys104, blue; Leu136, yellow; Arg218, pink; Lys276, green; **(B)** Delta Loop 2 in a single GlyR subunit. Residues interacting with Loop 2 are rendered as colored stick models: Lys104, blue; Leu136, yellow; Arg218, pink; Lys276, green (*Figure modified from Perkins et al. 2009*) (*Perkins et al., 2009*).