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STRUCTURAL MODELS OF LIGAND-GATED ION CHANNELS: SITES OF ACTION FOR ANESTHETICS AND ETHANOL

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Abstract

The molecular mechanism(s) of action of anesthetic, and especially, intoxicating doses of alcohol (ethanol, EtOH) have been of interest even before the advent of the Research Society on Alcoholism. Recent physiological, genetic, and biochemical studies have pin-pointed molecular targets for anesthetics and EtOH in the brain as ligand-gated ion channel (LGIC) membrane proteins, especially the pentameric (5 subunit) Cys-loop superfamily of neurotransmitter receptors including nicotinic acetylcholine (nAChR), GABA_A (GABA_AR), and glycine receptors (GlyR). The ability to demonstrate molecular and structural elements of these proteins critical for the behavioral effects of these drugs on animals and humans provides convincing evidence for their role in the drugs' actions. Amino acid residues necessary for pharmacologically relevant allosteric modulation of LGIC function by anesthetics and EtOH have been identified in these channel proteins. Site-directed mutagenesis revealed potential allosteric modulatory sites in both the trans-membrane domain (TMD) and extracellular domain (ECD). Potential sites of action and binding have been deduced from homology modeling of other LGIC with structures known from crystallography and cryo-electron microscopy studies. Direct information about ligand binding in the TMD has been obtained by photoaffinity labeling, especially in GABA_AR. Recent structural information from crystallized procaryotic (ELIC and GLIC) and eukaryotic (GluCl) LGICs allows refinement of the structural models including evaluation of possible sites of EtOH action.

Keywords

GABA_A receptors; GluCl pentameric ion channels; ELIC; GLIC; Loop 2; ethanol sites of action

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The subject of this mini-review has been presented in a symposium held at the Research Society on Alcoholism (RSA), June 23 to June 27, 2012 (San Francisco, California). Organizers and Chairs of the symposium were Richard W. Olsen and Daryl L. Davies. Introducer was Richard W. Olsen. Speakers were James R. Trudell, Keith W. Miller, Richard W. Olsen and Daryl Davies. Discussant was Daryl L. Davies.

INTRODUCTION

New homology models of GLYR AND GABA_AR provide near atomic level resolution and are suitable for studies of gating motions with molecular dynamics

In just the last few years, the structures available as templates for homology models of the glycine receptors (GlyR) and GABA_A receptors (GABA_AR) have improved dramatically. Previous cryo-electron microscopy structures of nAChR (Unwin, 2005) were at no better than 4 Angstrom average resolution which did not allow assignment or position of amino acid side chains nor water molecules or lipids. The recent X-ray structures of the prokaryotic GLIC and ELIC (Hilf and Dutzler, 2009; Nury et al., 2011; Howard et al., 2011; Spurny et al., 2013) and the eukaryotic GluCl (Hibbs & Gouaux, 2011) pentameric ion channels provide high homology to GlyR and GABA_AR and eliminate the troublesome loop connecting TM3 with TM4. The resulting homology models now allow precise identification of both agonist-binding and modulatory ligand-binding sites. As expected for these low affinity ligands, their binding is promiscuous and we identify multiple sites. We now (Murail et al., 2011) are using molecular dynamics simulations to estimate the relative populations of ligands at each site. However, we would also like to know the efficacy of the ligands at each of these sites. The goal is to extend these simulations to time scales sufficient to measure the effects of ligands on molecular motions, especially those involved in the potentiation of the opening transitions of these channels (Howard et al., 2011).

Several important points have emerged from recent studies using molecular modeling, molecular dynamics, mutagenesis, and X-ray crystallography; It is likely that the effect of small ligands will be revealed by experiments that explore the dynamics of the gating process. As the resolution of X-ray and molecular dynamics studies increases, we can expect new knowledge about the competition of anesthetics/alcohols with loosely bound water and lipid molecules (Trudell & Harris, 2004). Additionally, there is increasing evidence for a summation of potentiating and inhibiting binding sites in ligand-gated ion channels, often leading to apparent low binding constants and non-sigmoidal dose-response curves. Examples of distinct binding sites with antagonistic efficacies have been reported in nAChR (Borghese et al., 2003), GlyR (Crawford et al., 2007; Perkins et al., 2012), and GLIC (Howard et al., 2011).

Functional alcohol binding sites

Much of our direct evidence comes from LGICs other than GABA_ARs. The Cys-loop receptors consist of five subunits arranged in a roughly centrosymmetric fashion around a central ion conducting pore or channel. Each subunit has three major structural domains. The N-terminal extracellular, agonist-binding domain of some 200 residues (ECD), is followed by a domain of four trans-membrane alpha helices (TMD), interrupted by an intracellular domain of variable length between the third and fourth helix (ICD). Recent X-ray structural studies have shown remarkable sequence and structural homology among members of the Cys-loop pentameric LGIC superfamily. As a result, it is legitimate to use these systems to establish principles that may be applied to EtOH and GABA_ARs. For example, time-resolved photolabeling of azioctanol in *Torpedo* nAChR establishes that the affinity of azioctanol for its site increases in the order Resting < Desensitized < Open state (Forman & Miller, 2011). The models in Fig. 1 and 2AB were built by threading the GABA_AR sequence onto the recent high-resolution X-ray structure of a eukaryotic glutamate-gated chloride channel (GluCl, Hibbs and Gouaux, 2011) and the crystal structure of the prokaryotic GLIC channel (Nury et al., 2011), respectively. The global tertiary structures, and even the orientation of specific amino acid residues is essentially the same as earlier models of nAChR based on cryo-electron microscopy (Unwin et al., 2005).

The function of any protein that contains a pocket large enough to admit EtOH might in principle be modulated by EtOH. Using a published survey of pocket size in soluble proteins of known structure, some 15 – 20% of the sample contained pockets large enough to admit EtOH (Eckenhoff, 2001), a fact that is at odds with the number of known targets for EtOH. Two additional factors control the outcome of such binding. First, the affinity of the site for EtOH must be sufficient for occupancy to occur at concentrations attained by imbibing alcohol (10–30 mM)(Wallner et al., 2006; Murail et al., 2011). Second, there must be a mechanism by which such binding alters the function of the protein (Harris et al., 2008).

Many factors contribute to a drug's ability to interact with a site (Bissantz et al., 2010). The sum of the free energies of all these factors must be negative (favorable). Free energy must be provided to take EtOH from its aqueous environment and this free energy must be more than returned when EtOH binds to a pocket in a protein. The cost of removing EtOH from water is a constant for any putative binding site, so we may focus on the factors that make a site hospitable to EtOH. The ethyl group can contribute by interacting with the protein through van der Waals interactions, which are so short range (3.5 – 4.25 Å) that it follows that the pocket must have just the right geometry to ensure a close fit. Replacing a hydrogen atom with a methyl group in a drug has a small effect most of the time, but in 8% of the structures reviewed a 10-fold effect was achieved. This enhancement only occurred when the methyl group was buried in the interior of the protein with optimal methyl – side chain interaction distances (Leung et al., 2012). EtOH's hydroxyl can contribute one or two hydrogen bonds with the protein. This is essential, but note that EtOH lost similar hydrogen bonds when it was removed from water so the net free energy change may balance out (Trudell and Harris, 2004). In one crystallographic study of EtOH bound to a high affinity site on a protein used by flies to detect and avoid EtOH, the hydrogen bonds appeared to be particularly strong and susceptible to minor changes in the binding pocket (Thode et al., 2008).

Finally, if EtOH displaces a ligand from the binding pocket (most likely a water, but possibly a lipid) the unfavorable entropic cost of confining a freely roving solvated EtOH molecule in a tightly fitting binding pocket will be offset by the release of the native water (or lipid) (Trudell and Harris, 2004; Harris et al., 2008). To study EtOH binding in this detail in ligand-gated ion channels will require protein structures of a quality that has yet to be achieved in membrane proteins. Meanwhile advances in theoretical modeling detailed elsewhere in this review offer an effective approach (Howard et al., 2011; Murail et al., 2011).

For an alcohol to act on a protein it must either sterically occlude a functional region or it must stabilize one of the structural conformations that the protein undergoes while functioning. In the LGIC family, long chain alcohols do inhibit conduction by binding in the ion pore of cationic channels (for example, nAChRs), but EtOH is too small to do so (Borghese et al., 2003). Instead it shifts the agonist concentration-response curve to the left (Wood et al., 1991). This enhancing action is not on the agonist-binding step but is on the closed to open transition that occurs after the agonist binds; the probability that the channel will be open is increased by EtOH. This action is exerted through a different binding site from the inhibitory site of the longer chain alcohols, which photolabeling shows to be in the extracellular end of the ion-conducting pore (Pratt et al., 2000). In anion channels of the same superfamily (glycine and GABA_ARs in particular), all the alcohols act like EtOH (Mihic et al., 1997; Forman and Miller, 2011). This may be because the residues lining the extracellular end of the pore of the anionic channels are much more polar than those in the cationic channels, thus decreasing the affinity of the inhibitory site relative to that of the enhancing site.

EtOH can only have higher affinity for the open state of a channel relative to the resting state if its binding site changes its geometry during the transition between the two states. Unfortunately, this observation only restricts the possibilities slightly because gating is a concerted conformational change; changes originate at the agonist-binding site in the extracellular domain and end in opening the transmembrane ion channel over 50 Å away. What is known from years of study of allosteric mechanisms on other proteins is that effective sites tend to be found at the boundaries between either subunits or the separate structural domains within subunits and less often within a single domain (Cui and Karplus, 2008). Examples of all of these possibilities have been found in Cys-loop pentameric LGIC.

There is no crystal structure of a single LGIC in two conformations and there are no crystal structures of the mammalian neuronal heteropentameric Cys-loop superfamily of LGIC. The closest thing is the homopentameric nematode GluCl (Hibbs and Gouaux, 2011); the trimeric and tetrameric LGIC have totally different structures, and the possible TMD sites for anesthetics and alcohol are not well characterized. One of the most effective ways of locating and then characterizing a site as allosteric has been photolabeling. Quantitative photolabeling requires large amounts of material and has been limited to readily available sources such as the muscle type nAChR in *Torpedo*, but recently some success has been achieved with over-expression of recombinant heteromeric human GABA_ARs and this holds promise for EtOH studies in the future (Dostalova et al., 2010; Chiara et al., 2012; 2013).

On the nAChR, an inhibitory site for alcohols is located at the extracellular end of the channel at M2–20' (shorthand numbering of helical residues starting at the consensus N-terminal end of each segment) on the α subunit (E260) (Pratt et al., 2000). This site is not photolabeled in the resting state but is photolabeled in the open state milliseconds after adding acetylcholine. This is an allosteric site between subunits in the sense that the channel is the point where all five subunits meet and where a conformational change takes place to allow ions to flow through the pore. With the general anesthetic azi-etomidate, the same channel site was photolabeled in the open state. In addition, an intra-subunit site was detected in the helix bundle formed by the four transmembrane helices of the nAChR δ -subunit (Chiara et al., 2009). A smaller probe did not photolabel this region in the resting state, but did so within a millisecond of adding agonist to open the channel (Arevalo et al., 2005). These findings are of particular interest to alcohol researchers because this is the type of site that EtOH has been hypothesized to act on in the human GlyR and GABA_AR (Mihic et al., 1997; Wallner et al., 2006; Perkins et al., 2010). Recent technical advances have permitted the application of photolabeling to the human GABA_AR expressed in mammalian cells (Dostalova et al., 2010). Using these recombinant $\alpha 1\beta 3$ GABA_ARs, the hypothesis based on work with purified GABA_AR from bovine brain, that azietomidate has a binding site between the $\alpha 1$ - and $\beta 3$ - subunits in the TMD of the GABA_AR (Li et al., 2006), was confirmed and the extent of the site defined (Chiara et al., 2012). Etomidate causes general anesthesia at a few micromolar, whereas EtOH causes inebriation at 10–20 mM, so technical problems remain in applying such techniques to the EtOH site, but progress can be expected. It will be interesting to see where the high affinity sites for alcohols are and the degree to which they conform to the theoretical framework expounded in this review.

General anesthetic action sites in the TMD including long chain alcohols vs. specifically EtOH

Earlier mutagenesis work on long-chain alcohols including high dose (60 mM) EtOH and volatile and intravenous anesthetics by Mihic et al. (1997) revealed an intra-subunit pocket in GlyR and GABA_AR α and β subunits. Two residues in a given α or β subunit were identified at M2–15' and M3–4'. Other workers using mutagenesis implicated other residues in the transmembrane domain (TMD) for action of the GABA-enhancing neuroactive steroid

anesthetics (Hosie et al., 2006) but their structural model of a putative binding site(s) was subsequently shown to be incorrect[see below]. A collaborative team of R. Olsen, J. Cohen, K. Miller and colleagues used photoaffinity labeling with azi-etomidate to directly identify residues in the etomidate binding site of mg quantities of purified brain GABA_AR, defining a TMD inter-subunit binding site at the β +/ α - interface. The two residues labeled were α M1-11' and β M3-4' (Li et al., 2006; Chiara et al., 2012; Fig. 2). Fig. 2C shows a helical wheel diagram for the TMD helices of the multi-subunit GABA_AR channel protein, refined by cysteine replacement mutagenesis and sulfhydryl cross-linking on GABA_AR to properly stratify the helices (Jansen & Akabas 2006; Bali et al., 2009). The two etomidate residues (α M1-11' = M236 and β M3-4' = M286) are positioned sufficiently near each other to participate in a single binding pocket.

Note that the β M3-4' (M286) corresponds to one of the residues identified by Mihic et al. (1997) for possible intra-subunit binding sites for long chain alcohols and volatile anesthetics. The other residue in Mihic et al. (1997), M2-15' (β N265), was not identified by azi-etomidate affinity labeling, although this could be due to many reasons and does not prove it is not part of the binding pocket. The helical wheel diagram shows that this residue (β M2-15' = N265) is at the boundary of the intra- and inter-subunit sites and is not very far away from the etomidate pocket (Fig. 2). Plus, it is possible that the helices are not totally rigid and/or that the protein is flexible in this region in its native environment. Prior in vitro mutagenesis evidence showed that this residue (β M2-15' = N265) explains GABA_AR β subunit selectivity in etomidate modulation. In vivo evidence for importance of this residue in etomidate and propofol action was provided whereby a point mutation of this residue in the β 2 or β 3 GABA_AR mouse subunit produced a knock-in mouse that was insensitive to the sedative or anesthetic action, respectively, of etomidate and propofol (Rudolph & Antkowiak, 2004). This functional importance does not necessarily require the amino acid to be part of the ligand contact point; it could be allosterically coupled to anesthetic functional effects. However, cysteine replacement mutagenesis of the two residues identified by Mihic et al. (1997), M3-4' and M2-15' indicate that the former, but not the latter could be modified by irreversible sulfhydryl reagents that inactivated propofol modulation of function in a propofol-protectable manner (Bali & Akabas 2004). Likewise etomidate binding did not protect this residue from sulfhydryl inactivation of etomidate functional modulation of the cysteine substitution mutation (Desai et al., 2009). One conclusion proposed is that the M2-15' residue is allosterically coupled rather than part of the anesthetic binding pocket. In contrast, mutation of M2-15' to cysteine and covalent labeling with sulfhydryl reagents produced a GlyR ion channel with potentiated response to glycine that was largely identical to a GlyR exposed to alcohol or inhalational anesthetics, supporting the opposite conclusion that M2-15' is an important residue in an alcohol/anesthetic binding site (Mascia et al., 2000). Its sensitivity to modulators of heterogeneous chemical structures is probably more consistent with an allosteric coupling site. It should be emphasized that this EtOH interaction in the TMD involves anesthetic concentrations (60 mM), higher than those EtOH concentrations achieved in social drinking with anxiolytic and socializing effects, and the legal limit (17 mM in most states) for driving a car.

Azi-etomidate binding to the two inter-subunit site residues α M1-11' and β M3-4' (Li et al., 2006) was inhibited by etomidate, and allosterically enhanced by GABA and steroid anesthetics (Li et al., 2009). This shows the steroid site is not coincident with or overlapping with the etomidate site, further, the etomidate site (Fig. 2) proposed at the β / α interface site is validated, and the steroid cannot bind to the proposed α / β interface site because the residues are not sufficiently close (Hosie et al., 2006). Etomidate binding is allosterically inhibited by propofol, barbiturates, and volatile anesthetics, but not by anesthetic alcohols or EtOH at any concentration (Li et al., 2010; Olsen & Li, 2011). Interestingly, affinity labeling of recombinant α 1 β 3 γ 2 GABA_AR with an anesthetic barbiturate analogue (Chiara et al.,

2013) revealed high affinity binding to similar residues as etomidate but located at the α +/ β - and γ +/ β - interfaces. These sites had low affinity for etomidate while barbiturates had low affinity for the etomidate sites and allosterically inhibited them. This shows an emerging conclusion that the potential anesthetic modulatory sites have specificity for different chemical modulators in different subunit combinations.

Pharmacologically relevant high affinity sites for EtOH modulation on the extracellular domain of δ subunit-containing GABA_A receptors

Low dose (30 mM) EtOH enhances recombinantly expressed δ subunit-containing subtypes of GABA_AR, as well as tonic inhibitory GABA_AR-mediated currents in cells that express the extrasynaptic δ -GABA_AR (Wallner et al., 2006). Korpi et al. (1993) found a point mutation-- or allelic variant-- in the GABA_AR α 6 subunit (R100Q) in a rat strain that exhibited in vivo behavioral hypersensitivity to benzodiazepines (BZ) and also EtOH. The BZ sensitivity was explained by the α 6R100Q mutation, which gave high affinity BZ enhancement when expressed with β and γ 2 subunits in vitro in oocytes. Neither wild type nor mutant receptors showed enhancement by EtOH (up to 100 mM) when expressed with β and γ 2 subunits. Hancher et al. (2005) revisited this mutation and found that α 6R100Q, when expressed with β and δ subunits, gave even higher sensitivity to EtOH enhancement (low mM concentrations) in oocytes than the already sensitive α β δ subtype. The amino acid α 6R100 is located in the binding site for traditional BZ including the imidazobenzodiazepine EtOH antagonist drug Ro15-4513, and many behavioral effects of EtOH and the in vitro enhancement of GABA_AR in neurons, in culture, and in recombinant expression cells are reversed by Ro15-4513 (Wallner et al., 2006). They demonstrated that Ro15-4513 not only blocks low mM EtOH enhancement of δ -GABA_AR currents, but this action is reversed by the close structural analog flumazenil (Ro15-1788), the traditional BZ antagonist. A minor subset of BZ ligands, notably imidazobenzodiazepines and β carboline compounds, are able to mimic or inhibit Ro15-4513 activity on EtOH in vivo and in vitro, and the same ligands, and only those, are able to inhibit the binding of [³H]Ro15-4513 to native and recombinant δ -GABA_AR. Whereas it had been believed that no BZ ligands bound to the δ -GABA_AR subtypes, this new evidence suggests that δ -GABA_AR have a novel BZ/ EtOH site with unique pharmacology with respect to BZ ligands (Wallner et al., 2006). Thus, this work defines an EtOH receptor, when it was not clear that there is such a thing in the traditional sense! Note that some workers (Linden et al., 2011) propose alternative interpretations for these observations suggesting that BZ ligands like Ro15-4513 acting on γ -GABA_AR produce behavioral excitation which indirectly antagonizes EtOH's low-moderate dose behavioral depression produced at non-GABA_AR targets, or at least non- δ -GABA_AR targets. Further mutagenesis of residues in the extracellular BZ binding domain (ECD) of α 6 β δ -GABA_AR revealed preliminary evidence for an EtOH-sensitive Ro15-4513 binding site at the α +/ β - interface (Fig. 3). Starting with sequence loops in the major subunit for this binding site (α), including α 6R100, we predicted sequences for the minor subunit potentially situated abutting this α pocket domain, which could be either on δ or on β . Mutagenizing residues in the sequence of the δ subunit, e.g., H68A (Meera et al., 2010) imparts classical BZ site binding to the heteropentamer, but does not disturb the EtOH-sensitive Ro15-4513 sites in δ GABA_AR. On the other hand, the selectivity of β subunits of the EtOH binding (β 3> β 2> β 1, Wallner et al., 2006), can be identified in preliminary mutagenesis studies to involve this loop (Fig. 3A) in the β subunit domain, 62–66 in β 3 (Wallner, Hancher, & Olsen, unpublished).

Thus an ECD site for EtOH modulation may resemble the traditional binding and resulting efficacy of ligands like agonists (e.g., GABA) and more well-understood modulators (e.g., BZ). Interestingly, the aziridine analogue of the anesthetic alcohol *n*-octanol photoaffinity labels residues in nAChR TM2 but also in the agonist site in the ECD (Pratt et al., 2000).

Recently, the anesthetic ketamine was found bound to the ECD domain homologous to the cholinergic antagonist site in nAChR by X-ray crystal structural analysis of GLIC (Pan et al., 2012); the anesthetic bromoform was found in X-ray crystal structures of ELIC at binding sites both in the TMD and at a hydrophobic pocket in the ECD between $\beta 7$ - $\beta 10$ strands (Spurny et al., 2013), also located near the agonist binding site.

Targets for EtOH action and sensitivity control are found in the extracellular domain loop 2 of GLYR and GABA_AR

A critical barrier to the development of effective therapies for alcohol use disorders (AUDs) has been our lack of knowledge regarding the molecular targets upon which EtOH acts in the brain to cause intoxication, tolerance, reward and dependence. To provide novel tools to address this issue, the Alkana/Davies group developed mutant receptors that are supersensitive to EtOH [for review see Perkins et al. (2010)]. To accomplish this, we took advantage of GlyRs and GABA_ARs, which differ significantly in their sensitivities to EtOH. For example, $\alpha 1$ GlyRs do not reliably respond to EtOH concentrations less than 20 mM. Similarly, γ subunit-containing GABA_ARs (e.g., $\alpha 1\beta 2\gamma 2$), the most predominantly expressed GABA_ARs in the CNS, are insensitive to EtOH concentrations less than 50 mM. In contrast, δ subunit-containing GABA_ARs (e.g., $\alpha 4\beta 3\delta$) have been shown to be sensitive to EtOH concentrations as low as 1 mM (Wallner et al., 2003, 2006b). Sequence alignment of $\alpha 1$ GlyR, γ GABA_AR, and δ GABA_AR revealed differences between the Loop 2 regions (Fig. 1A and B) and the primary sequences of these receptor subunits (Fig. 3).

Previous investigations in GlyRs indicate that position 267 in the transmembrane domain (TMD) domain and position 52 in extracellular domain loop 2 (L2) are part of the same alcohol pocket and that the net effect of EtOH on GlyRs reflects the summation of its respective positive and negative modulatory effects on these targets (Crawford et al., 2007). Moreover, replacing Loop 2 of $\alpha 1$ GlyR subunits with Loop 2 from the δ GABA_AR (δ L2), but not the γ GABA_AR subunit, reduced EtOH threshold and increased the degree of EtOH potentiation without altering general receptor function (Perkins et al., 2009). Similarly, replacing Loop 2 of the γ subunit of GABA_ARs with δ L2 shifted EtOH threshold from 50 mM in WT to 1 mM in the GABA_A γ - δ L2 mutant. These findings indicate that the structure of Loop 2 can profoundly affect EtOH sensitivity in GlyRs and GABA_ARs. Interestingly, the mutant receptors tested involved mutating all five of the non-conserved residues in Loop 2 of $\alpha 1$ GlyRs, and Loop 2 of the $\gamma 2$ subunit in GABA_ARs, to those found in the GABA δ subunit (Fig. 3).

Presently, we do not know which combination of mutations causes the increased EtOH sensitivity of δ L2 mutant GlyRs and GABA_ARs or if the δ L2 mutation produces the highest EtOH-sensitivity receptor possible. For example, we know that a point substitution of serine for alanine at position 52 (A52S) decreases EtOH sensitivity in $\alpha 1$ GlyRs (Mascia et al., 1996, Davies et al., 2004). Ironically, this mutation exists in the δ L2 substitution. Therefore, we hypothesized that restoring position 52 to alanine in the δ L2 mutant receptors would further enhance their sensitivity to EtOH. To test this, we substituted A52S back to A52 in δ L2 mutant GlyRs (δ L2 A52 GlyRs), expressed the mutant receptors in *Xenopus* oocytes, and tested them using two-electrode voltage clamp. As predicted, the δ L2 A52 GlyRs were more sensitive to EtOH—they had a greater magnitude of EtOH response and lower EtOH threshold (< 1mM)—compared to the δ L2 mutant GlyRs. On the other hand, we found that mutating position 52 in mutant δ L2 1GlyRs to glutamic acid (A52E) reduced the sensitivity of the receptor to EtOH (Perkins et al., 2012). Exploration of the physical-chemical parameters of the residue at position 52 indicate that charge interactions determined by the specific geometry of the amino acid at position 52, rather than molecular weight or volume, play differential roles in receptor sensitivity to agonist and EtOH.

Taken together, these results focusing on Loop 2 of GlyRs and GABA_ARs indicate that Loop 2 plays a key role in controlling EtOH sensitivity across the Cys-loop superfamily of receptors. Collectively, this knowledge will help to define the chemical architecture of this site of EtOH action for potential drug development and the key physical-chemical parameters that cause and control EtOH sensitivity in GlyRs, GABA_ARs, and possibly other Cys-loop family LGICs. In addition, the findings suggest that mutant ultrasensitive EtOH receptors (USERS) can be developed as novel tools to help identify roles that EtOH's actions on specific receptor subunit combinations play in causing the behavioral effects of EtOH and AUD. Once identified, these receptors can be targeted for new medication development.

SUMMARY AND CONCLUSION

Thus, EtOH at high concentrations (> 50 mM), along with long chain alcohols, may interact with the TMD inter- (Murail et al., 2011) and intra-subunit (Mihic et al., 1997) pockets of pentameric Cys-loop LGICs. Microsecond simulations indicate that EtOH binds between subunits and could stabilize an open-state model of a glycine receptor (Murail et al., 2011), a suggestion supported by crystal structures of prokaryotic homopentameric LGICs (Nury et al., 2011; Howard et al., 2011; Spurny et al., 2013). The low dose EtOH (~50mM) sites of action, however, may involve a type of BZ site in the ECD demonstrated for certain GABA_AR subtypes, and possibly in the GlyR ECD as well. It appears that the pentameric LGICs with heteromeric subunit composition have innate subunit-dependent effects on ligand affinity, including GABA, BZ, and other allosteric modulators. These sites at subunit interfaces could be pharmacologically more relevant for low affinity ligands such as anesthetics, etomidate, steroids, barbiturates (e.g., Chiara et al., 2013), and especially EtOH. Multiple classes of binding sites for this type of ligand will be described as technology advances, but most of them probably will not be pharmacologically important. That is, many promiscuous sites may be occupied but have no efficacy, while some may possess the critical efficacy. The question of whether these low affinity allosteric modulators actually follow the more traditional ligand binding with energetics sufficient for modulation of function of the pentameric ligand-gated ion channels will be addressed.

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Figure 1.

A homology model of GABA_Aα2β2γ2 was built by threading the primary sequence onto the template of the glutamate-gated chloride channel (GluCl, PDB ID 3RHW, Hibbs and Gouaux, 2011). (A) A view looking down the axis of the ion channel from the extracellular side; α2, β2, and γ2 subunits are rendered as solid ribbons and colored yellow, red, and green, respectively. Several residues mentioned in the text are rendered as space filling surfaces: beta M2 N265 and K274, in pink; M3 M286 and V290, in turquoise; alpha M1 M236, in turquoise; and Loop 2 P52-E59, with carbon, hydrogen, oxygen, and nitrogen in gray, white, red, and blue surfaces. (B) A view from the plane of the lipid bilayer towards the β3 – α1 subunit interface.

Figure 2.

Three views of the $\alpha 1\beta 2\gamma 2L$ GABA_A receptor showing residues photolabeled by etomidate derivatives. **A.** View from the extracellular side looking down the ion channel (light blue circle). Residues photolabeled by etomidate derivatives in the transmembrane domain between $\beta 3$ – and $\alpha 1$ – subunits have cyan carbons. For reference in the extracellular domain, the agonist site in the same interface is indicated by $\alpha 1$ Tyr-205 (~50 Å above the etomidate site) and the benzodiazepine site between the $\alpha 1$ – and $\gamma 2L$ subunits is indicated by $\alpha 1$ His-202. **B.** A view from the lipid bilayer towards the $\beta 3$ – $\alpha 1$ subunit interface. $\beta 3M3$ Met-286, $\beta 3M3$ Val-290 and $\alpha 1M1$ Met-236 are residues in the etomidate binding site (Li et al., 2006; Chiara et al., 2012). The orientation of residues is suggested by a homology model (Chiara et al., 2013) built on the GLIC crystal structure (Nury et al., 2011). Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from the National Institutes of Health (National Center for Research Resources grant 2P41RR001081, National Institute of General Medical Sciences grant 9P41GM103311). **C.** Helical wheel representation of the GABA_A β/α interface in the trans-membrane domain illustrating the proposed binding site for etomidate (modified from Li et al., 2009) with the residues in $\alpha 1M1$ and $\beta 3M3$ photolabeled by [3H]azetomidate (circled residues in *green*) contributing to a common binding pocket at the β/α interface. Also included in *pink* is the position in $\beta 3M2$ (N265) that functions as a determinant of etomidate/azetomidate anesthetic potency *in vivo* (Rudolph &

Antkowiak,2004), the residues in α M1 and β M3 identified as sensitivity determinants for direct activation by neurosteroids (boxed residues in *yellow* [Hosie et al., 2006]), and the positions in α M1 and β M3 that when mutated to Cys can form intersubunit cross-links (*red* and *orange*).

Figure 3. Sequence alignment of key alcohol site residues in GABA-R α and β subunits and in GlyR $\alpha 1$ subunits

(a) Sequence alignment of GABA-R “loop A” α subunits with the benzodiazepine-H/R/Q100 (in $\alpha 6$) binding site residues, colored; H (yellow); R (green) and the $\alpha 6$ -R100Q alcohol polymorphism (red) (Hanchar et al., 2005). Since this is a highly conserved region all the other amino acid residues shown are identical. (b) Sequence alignment of “Loop2” and “Loop D” comprising two beta sheet structures ($\beta 1$ and $\beta 2$, highlighted in yellow) in the acetylcholine binding protein (AChBP, Brejc et al., 2001), verified in the structure of the invertebrate LGIC, GluCl (Hibbs & Gouaux, 2011). Line 1 shows AChBP (with the nicotine binding residue W53 and Q55 boxed) from which structural homology models have been derived (Ernst et al., 2003). Three critical γ “loop D” BZ-site residues in the $\gamma 2$ subunit (F77, A79, T81) are boxed and colored green (Holden & Czajkowski, 2002). These three residues contribute to the classical BZ binding site at the $\alpha(1,2,3,5)+\gamma 2$ subunit interface (abutting the yellow H residue in Loop A shown in (a)). The three β subunits are identical in this region except for residues 66 (boxed, S in $\beta 1$, A in $\beta 2$ and Y in $\beta 3$ (red)). Also boxed in this alignment is residue δ H68 that confers diazepam sensitivity to highly EtOH-sensitive $\alpha 4\beta 3\delta$ receptors when changed to A, the residue present in $\gamma 2$ at the homologous position (Meera et al., 2010). In addition, we show the GABAR $\alpha 1$ sequence with four residues boxed: $\alpha 1$ -D57 is critical for the coupling of agonist binding to channel activation (Kash et al., 2003), and residues D62, F64, and R66 are critical residues for binding of the GABA antagonist Gabazine (Holden and Czajkowski, 2002); F64 is also critical for (affinity labeled by) muscimol binding (Olsen & Li, 2011). Finally, we show Loop 2 alcohol residues (box) from the GABA-R δ , $\gamma 2$, and the GlyR $\alpha 1$ subunit [blue (Perkins et al., 2009; Crawford et al., 2007)]. These loop 2 residues are flanked not only by ligand binding site residues in loop D (as shown in Fig. 3b), but also by residue Glu-Cl R37 (in loop G) just upstream in GluCl which contributes to glutamate binding sites in the Glu-Cl-glutamate co-crystal structure (see Fig.3b in Hibbs and Gouaux, 2011). Therefore homologous residues could -- depending on the size of the side-chain as well as the size and exact positioning of the ligand in the ligand binding site -- contribute to the ligand binding at receptor subunit interfaces.