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Alcohol dependence: molecular and behavioral evidence

James R. Trudell¹, Robert O. Messing², Jody Mayfield², and R. Adron Harris²

¹Department of Anesthesia, Stanford University School of Medicine, Stanford, CA 94305

²Waggoner Center for Alcohol and Addiction Research, The University of Texas at Austin, Austin, TX 78712

Abstract

Alcohol dependence is a complex condition with clear genetic factors. Some of the leading candidate genes code for subunits of the inhibitory GABA_A and glycine receptors. These and related ion channels are also targets for the acute actions of alcohol, and there is considerable progress in understanding interactions of alcohol with these proteins at the molecular and even atomic levels. X-ray structures of open and closed states of ion channels combined with structural modeling and site-directed mutagenesis have elucidated direct actions of alcohol. Alcohol also alters channel function by translational and post-translational mechanisms, including phosphorylation and protein trafficking. Construction of mutant mice with either deletion of key proteins or introduction of alcohol-resistant channels has further linked specific proteins with discrete behavioral effects of alcohol. A combination of approaches, including genome wide association studies in humans, continues to advance the molecular basis of alcohol action on receptor structure and function.

Keywords

ethanol; LGICs; GABA_A; NMDA; glycine; GLIC; crystal structures; PKC; trafficking; knockout; knockin mice; behavior; chronic alcohol consumption; gene expression

Alcohol's Molecular Targets

The pathway from an initial drink of alcohol (ethanol) to dependence is a long and complex one. However, in recent years there has been much progress in understanding the complexity and dynamicity of the acute and chronic mechanisms at play. This review describes the acute actions and chronic, persistent adaptations of alcohol, encompassing the structural, protein, intracellular, and genomic targets implicated in alcohol dependence. First, some of the rapid-onset effects of alcohol are likely mediated via direct action on ion channels [1]. The strongest evidence for the receptors most responsible for intoxication comes from the

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Corresponding author – Harris, R. A. (harris@austin.utexas.edu).

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family of Cys-loop pentameric ligand-gated ion channels (LGICs), including GABA_A, glycine, nicotinic cholinergic, and serotonin receptors, as well as other ion channels such as NMDA and G-protein-gated inwardly rectifying potassium (GIRK) [2]. Intoxicating concentrations of alcohol (10-25 mM) produce euphoria and decrease inhibitions, and approximately 17 mM is considered the legal definition of intoxication in the United States. Second, there are slow-onset adaptations to alcohol that may include phosphorylation of LGICs, interactions with intracellular messenger proteins, and alterations in channel proteins [3, 4]. Third, there are long-term adaptations in brain gene expression caused by chronic alcohol administration, and these changes in expression are organized into defined modules of related genes that are brain-region and cell-type specific [5]. However, alcohol-mediated changes in gene regulation do not necessarily explain changes in proteins, thus underscoring the necessity for a combination of rigorous genomic and proteomic approaches for a more complete understanding of the chronic effects of alcohol abuse and how best to approach its treatment [6].

Locating sites of acute alcohol action that are critical for modulating ion channel function was first facilitated by chimera and site-directed mutagenesis studies. Chimeras between the alcohol-inhibited GABA_A ρ receptor and the alcohol-potentiated GABA_A and glycine receptors provided initial evidence for transmembrane sites of alcohol/anesthetic action on LGICs. This important finding was followed by single-point mutations that localized the critical amino acid residues to the transmembrane domain of glycine and GABA_A receptors. Mutagenesis and covalent labeling studies suggested that these receptors were modulated by actions at competitive binding sites for alcohols and anesthetics [2, 7]. Subsequent studies of GLIC (a prokaryotic homolog of pentameric LGICs) provided a unique structural model for identifying both the potentiating and inhibitory effects of n-alcohols on LGICs and the corresponding amino acid residues involved [8]. Combining X-ray crystallography, structural modeling, and site-directed mutagenesis has further advanced resolution of alcohol binding pockets in diverse classes of brain proteins [2, 9]. Crystallography studies of the GIRK channel recently demonstrated that alcohol activation is mediated via a unique microprotein domain that is distinct from receptor-mediated channel gating [10], and the chemical properties revealed here might be relevant for other alcohol-sensitive proteins.

How does the small, weakly binding ethanol molecule affect the function of large proteins such as LGICs and other ion channels? An important point about weakly interacting ligands, such as alcohol, is that the term ‘binding’ should be used cautiously. Weber introduced the concept that the forward rate constant for binding of small molecules is diffusion controlled (approximately 10^7 /mol $\times$ sec), thereby allowing estimation of the unbinding rate for ethanol as less than one microsecond [11]. Although surprising, this result is within the timescale of electron paramagnetic resonance [12] and nuclear magnetic resonance studies that estimated the equilibration time of halothane into membranes [13]. These results are also consistent with a one-microsecond molecular dynamics simulation of ethanol molecules in a fully hydrated lipid membrane, showing ethanol hopping from site to site on a time scale of nanoseconds [14]. Thus, for alcohol and other weakly bound molecules such as inhaled anesthetics, the term “binding” more accurately refers to “probability of occupation.”

Given the low binding energy and low probability of occupation of an alcohol binding site, we must determine how alcohol acts once it transiently occupies a site such as an internal cavity of a receptor protein [1]. A cavity can open or collapse during the resting/open/desensitized cycle of ion channel function, and alcohol could act via one of three mechanisms: (1) occupy the cavity and prevent its collapse, (2) displace preexisting water molecules that are essential for the transitions, and (3) provide H-bond or van der Waals bridges that facilitate the transitions [2].

The transient presence and small size of alcohol in an internal cavity might be expected to have little effect on the static [15] or crystal structure of a protein [16]. However, alcohol molecules likely change the dynamics of LGICs [17, 18]. Ethanol sites can be visualized through crystal structures as occupying a water-filled cavity, and replacement of water by ethanol results in a higher resolution structure for GLIC [19] and was also important for crystallization of the alcohol-binding protein LUSH from *Drosophila* [20]. Thus, it appears that ethanol can stabilize specific states or conformations of these proteins, and for ion channels, this may correspond to open or closed states, providing a basis for changes in channel function [19]. This point is shown in Figure 1, where the B-factor, a measure of structural flexibility, is increased by the mutation and decreased by ethanol binding.

Crystal structures are not ideal for assessing dynamic changes, and computational approaches (molecular dynamics) may provide greater insight. However, at present, even a one microsecond molecular dynamics simulation of alcohol in a pentameric LGIC requires a large computer cluster and six weeks of computational time [8, 14]. Fortunately, this hurdle may be reduced by the identification of both the ‘resting’ and ‘open’ states of the same GLIC channel [8, 19]. In contrast to the very slow ‘random walk’ of molecular dynamics between two states, the transition between two known conformations of the same receptor can be simulated much faster [17, 21], thereby facilitating study of the transient binding of alcohol.

The choice of GLIC for resolving structures is particularly apt because the initial structure has been used for molecular dynamics and homology modeling of other LGICs [17, 22]. Although GLIC is relatively insensitive to alcohols, a point mutation in the porelining α helix was shown to confer alcohol sensitivity [8], thereby making it an ideal template for building homology models of LGICs. Moreover, comparison of x-ray structures of this mutant crystalized with and without alcohols identified the properties and location of residues that are key for an alcohol-binding site. Crystallography of the ethanol-sensitive mutant GLIC channel showed a water-filled cavity located between α helices and identified the hydroxyl group of ethanol forming a hydrogen bond with asparagine as a critical mechanism for alcohol modulation of channel function (Figure 1) [19]. Structural motifs conferring alcohol sensitivity may be conserved between homologs from lower organisms and mammalian LGICs. Structural resolution and computational modeling of alcohol binding sites combined with mutagenesis studies have proven to be powerful tools for identifying alcohol-binding sites in LGICs. For a timeline of events characterizing the structural refinement of pentameric LGICs and ethanol-sensitive models, see Table 1 [2].

Translating Protein Interactions Into Behavior

The structural studies discussed above clearly demonstrate molecular sites where ethanol can interact with key brain proteins, particularly ion channels, to alter their function. This raises the key question of which (if any) of these target proteins account for specific behavioral actions of ethanol. Two widely used approaches to link specific proteins with behavior are genetic deletion of a protein in a null mutant or knockout mouse and viral delivery of inhibitory RNAs or other RNAs to produce knockdown in specific brain regions. The knockout strategy has been used extensively in alcohol research [23, 24] and local knockdown was also used for specific GABA_A receptor subunits [25, 26]. An alteration of behavioral effects of alcohol resulting from these manipulations implies that the manipulated protein is involved, but does not demonstrate that alcohol acts on the protein to produce the effect. Thus, removal of a protein does not define whether the behavioral change is due to a direct action of ethanol on the protein or another role of the protein in the behavior; another limitation of knockout animals is the potential compensatory role of other proteins, which could complicate interpretation of behavioral results. A more direct approach for linking ethanol-sensitive sites on proteins to behaviors involves the construction of knockin animals, where the normal protein is replaced by one in which the alcohol site is mutated to prevent alcohol action. The goal is to remove alcohol action on a single protein without altering the other functions of that protein but this approach, like knockout models, could be compromised by compensatory mechanisms from other proteins. The knockin strategy has been employed with the $\alpha 1$ and $\alpha 2$ subunits of GABA_A receptors and showed a rather selective linkage of $\alpha 1$ with alcohol tolerance and physical dependence and $\alpha 2$ with alcohol consumption, taste aversion, and motor stimulation [27]. Knockin mice with ethanol-resistant NMDA receptors (GluN1 subunit) also showed specific changes in alcohol behaviors with decreased anxiolytic responses, but no changes in several other behaviors [28]. These studies provide evidence that ethanol action on specific GABA_A and NMDA receptor subunits are responsible for some of the distinct behavioral effects of ethanol. One caveat of this approach is that mutations that eliminate ethanol action may also change the function of the protein, thus accounting for the altered behavior. For example, a gain-of-function mutation in the GABA_A $\beta 1$ subunit altered alcohol consumption independent of ethanol's actions on the receptors [29]. Despite the excellent structural studies, the glycine receptor (GlyR) $\alpha 1$ subunit has proven very difficult to engineer with normal glycine sensitivity and reduced ethanol action. Two different knockin mouse lines were constructed with ethanol-resistant mutations in the GlyR $\alpha 1$ subunit, but both lines showed impaired glycine receptor function, which caused these mutations to be homozygous lethal [7, 30]. The heterozygotes were viable and were tested for behavioral actions of ethanol but also demonstrated reduced glycinergic inhibition, complicating interpretation of the results.

It is unlikely that the receptors studied to date using the knockin strategy will account for all, or even most, of the important brain alcohol targets responsible for alcohol behaviors. One approach for prioritizing ion channels is genetic association with human alcohol dependence. Such studies nominated the GABA_A $\alpha 2$ subunit, which has already been studied in mutant mice, as a top candidate [31]. An extensive meta-analysis of GABA_A receptor genes identified a strong association of *GABRA2* (GABA_A $\alpha 2$) with alcohol

dependence as well as evidence for association of *GABRG2* (GABA_A γ 2) and *GABRA6* (GABA_A α 6) [32]. A role for the GlyR α 3 subunit has also been reported [33], providing a genetic rationale to extend the studies of GlyR α 1 to the α 2 and α 3 subunits. There is also a neurobiological rationale for studying GlyRs. For example, the supraspinal GlyRs found in reward-related pathways [34] may contain α 2 or α 3 subunits [35, 36]. The function of GlyRs is enhanced by ethanol for both recombinant and native receptors, and this action can be obtained with pharmacologically-relevant concentrations (20 mM and above) [7, 37, 38]. In a recent examination of the mechanism of alcohol inhibition of lateral orbitofrontal cortex neurons, GABA receptors made no contribution to the inhibitory effects of ethanol and there was only a minor role for glutamate receptors, with most of the inhibition due to enhancement of GlyR function [37]. This study also demonstrated that the ethanol effects were brain-region and cell-type specific. Other members of the Cys-loop LGICs with genetic linkage to alcohol dependence include the GABA_A ρ 1 and ρ 2 subunits [39]. GABA_A ρ subunits are found in many brain regions and may contribute to synaptic and extrasynaptic GABA responses as well as gliotransmission. Ethanol inhibits the function of these receptors and genetic deletion of the GABA_A ρ 1 subunit decreased ethanol consumption and allowed faster recovery from motor incoordination, suggesting a role in these behavioral effects of ethanol [40]. Another promising candidate is the GIRK2 channel, which has been linked to alcoholism in humans [41, 42] and alcohol actions in null mutant mice [43]. There is also structural information about the mechanism of alcohol action on this protein [10]. It is likely that *in vivo* manipulation of alcohol binding sites on ion channels such as glycine, GABA_A ρ , and GIRK will further disclose other proteins important for alcohol-mediated behaviors [2].

Ethanol Modulation Via Receptor Phosphorylation

Although there is structural, molecular, and behavioral evidence for direct actions of alcohol on ion channels, alcohol can also indirectly regulate ion channel function (and presumably behavior) by altering post-translational processing or protein trafficking. For example, ethanol alters the function of the related non-receptor protein tyrosine kinases Src and Fyn, which modulate NMDA receptors and synaptic plasticity [44]. Ethanol inhibits Src in hippocampal neurons, causing internalization of the GluN2A subunit of NMDA receptors [45]; this process alters subunit composition, leading to receptors containing mainly GluN2B subunits. In addition, ethanol stimulates Fyn-mediated phosphorylation of GluN2B, thereby enhancing channel function and contributing to acute tolerance to ethanol [46]. Repeated administration of ethanol increases Fyn-mediated phosphorylation and synaptic localization of GluN2B and produces long-term facilitation of NMDA receptor function in the dorsomedial striatum [47, 48], a brain region important for learning and expression of goal-directed actions. In addition, administration of inhibitors of Src or GluN2B-containing NMDA receptors into the dorsomedial striatum decreases operant self-administration of ethanol and reinstatement of ethanol seeking [48]. Thus, ethanol activation of Fyn, specifically within this brain region, enhances NMDA receptor signaling in a manner that facilitates increased ethanol intake and relapse. The mechanism may involve recruiting protein tyrosine phosphatase α (PTP α) to synaptic membranes, where it dephosphorylates

Fyn at tyrosine 532, leading to Fyn activation [49]; however, how ethanol recruits PTP α to synapses is unknown.

Ethanol responses at GABA $_A$ receptors are also regulated by phosphorylation. Protein kinase C (PKC ϵ) reduces the positive allosteric effects of benzodiazepines and ethanol at GABA $_A$ receptors by phosphorylating $\gamma 2$ subunits at Ser-327 [50], and mice lacking PKC ϵ (*Prkce*^{-/-} mice) are supersensitive to the intoxicating effects of ethanol and benzodiazepines [51]. The recent discovery of an endogenous, positive, benzodiazepine-like, allosteric modulator of GABA $_A$ receptors [52] raises the possibility that PKC ϵ modulates sensitivity to endogenous positive GABA $_A$ receptor modulators and thereby regulates reward signaling [53]. Such a hypothesis is consistent with studies of ethanol consumption and reward in *Prkce*^{-/-} mice. These mice drink substantially less alcohol than wild type [51] and show heightened aversion to alcohol [54]. In addition, ethanol stimulated GABA release in PKC ϵ ^{+/+} neurons from mouse central amygdala but not in PKC ϵ ^{-/-} neurons, and these findings were confirmed using a specific PKC ϵ inhibitor [55].

In contrast to *Prkce*^{-/-}, mice lacking the related PKC δ show resistance to ethanol intoxication [56]. This response may be related to ethanol actions at GABA $_A$ receptors that contain δ subunits and mediate tonic GABA inhibition, rather than at receptors that contain $\gamma 2$ subunits and mediate synaptic GABA currents. Tonic currents in thalamic and hippocampal neurons from *Prkcd*^{-/-} mice show no response to 30 mM ethanol, whereas tonic GABA currents in neurons from wild type mice are enhanced by ethanol [56]. Furthermore, in mouse L(tk-) fibroblasts that express $\alpha 4/\beta 3/\delta$ GABA $_A$ receptors, ethanol enhancement of GABA currents is also enhanced by PKC δ [56]. These findings suggest that PKC δ promotes ethanol intoxication by enhancing ethanol's action at extrasynaptic GABA $_A$ receptors, possibly through phosphorylation of δ subunits. As discussed previously, knockout animals do not provide direct evidence for a role of the protein in alcohol-mediated behaviors and do not account for compensatory mechanisms. Identification of PKC phosphorylation sites and construction of corresponding knockin animal models will be important to provide more direct evidence for a role of PKC subtypes in mediating the behavioral effects of alcohol.

Ethanol Modulation of Receptor Trafficking

Ethanol activates signaling pathways that induce receptor trafficking and can change the composition of receptors at synapses and extrasynaptic sites. For example, a single intoxicating dose of ethanol rapidly decreases extrasynaptic GABA $_A$ receptors that contain $\alpha 4$ and δ subunits in hippocampal pyramidal neurons, which may contribute to acute tolerance to ethanol [57, 58]. Several hours later, there is also reduced synaptic GABA $_A$ receptor function that is due to internalization of $\alpha 1$ subunits, a shift in $\alpha 4$ subunits from extrasynaptic to synaptic sites and an increase in $\gamma 2$ subunits, resulting in the appearance of synaptic receptors containing $\alpha 4$ subunits that carry currents of shorter duration than synaptic receptors containing $\alpha 1$ subunits [57]. These acute effects of ethanol are reversible after a single exposure, but with chronic intermittent exposure leading to ethanol dependence, they become irreversible. Similar findings have been reported in basolateral amygdala after chronic ethanol exposure, although the changes in receptor subunits are synapse-specific, with reductions in $\alpha 1$ subunits occurring beneath inputs from lateral

paracapsular amygdalar neurons and increases in $\alpha 4$ beneath inputs from local interneurons [59]. It is interesting that interneuronal expression of $\alpha 1$ -containing GABA_A receptors was implicated in the addictive properties of benzodiazepines [60], and such cell-type and subunit-specificity is also likely important in the addictive profile of alcohol on these and other LGICs.

How ethanol evokes changes in receptor trafficking is not clear. Evidence suggests that rapid downregulation of δ subunits is due to increased binding of δ subunits to clathrin AP2- $\mu 2$ and subsequent endocytosis [61], but the mechanism of ethanol-induced binding is not known. PKC γ may also play a role in trafficking. In cultured rat cortical neurons, PKC γ co-immunoprecipitates with GABA $\alpha 1$ and $\alpha 4$ subunits [62]. Treatment with short-interfering RNAs targeted against PKC γ prevents ethanol-induced decreases in $\alpha 1$ subunits and increases in $\alpha 4$ subunits in synaptosomal (P2) fractions prepared from cultured cortical neurons [63]. However, these results should be viewed as preliminary because three siRNAs were used against PKC γ and they appear to have been administered together, not separately, and without rescue, it is not possible to exclude off-target actions of the siRNAs.

Overall, a range of different approaches provides evidence that alcohol action is controlled, in part, by phosphorylation and other post-translational modifications of LGICs, particularly GABA_A receptors. Future studies will be needed to identify the kinase substrates and chaperones that mediate these actions of alcohol and to examine receptor mobility between synaptic and extrasynaptic sites within the plasma membrane. Synaptic plasticity, both long-term potentiation and long-term depression, can be influenced by receptor number and subunit composition, and alcohol-induced changes in populations of proteins (e.g., LGICs) could produce persistent neuroadaptations that affect neuronal activity, cell survival, and contribute to the addictive properties of this drug.

Concluding Remarks

Convergent results from a number of structural and computational approaches show that alcohol can occupy discrete water-filled cavities in LGICs and thereby alter their function. Cys-loop receptor homologs from lower organisms have proved particularly valuable in modeling structural and functional channel properties in this family in unprecedented detail. A major limitation in characterizing ethanol modulation of LGICs at a molecular level is the absence of high-resolution structural data for human receptors in this superfamily. Global gene deletion studies have provided valuable information on protein targets that may be important in alcohol dependence. However, the use of knockin mice with mutations that render specific LGICs resistant to alcohol provides a more powerful technique to link direct actions on proteins with behavioral effects. These actions are rapid and reversible, but alcohol exposure *in vivo* also produces longer-term adaptations, including receptor phosphorylation and trafficking, which may be more persistent and relevant to neuroadaptations mediated by chronic alcohol consumption such as tolerance and dependence. Advances in neuroproteomics combined with other approaches will further advance alcohol research by integrating changes in protein structure, function, interactions, and networks, with the potential to identify relevant biomarkers for diagnosis and pharmacological targets for intervention [6]. Much work has focused on GABA_A and

glycine receptors, in part, because of detailed structural information from related bacterial LGICs such as GLIC and, in part, because of genetic linkage of these receptors with human alcohol dependence.

However, we must also consider other relevant targets of alcohol action in brain that genomic and proteomic studies bring to the forefront. Several genes of alcohol-metabolizing enzymes (alcohol and aldehyde dehydrogenases) are strongly associated with the pathogenesis of human alcoholism in genome wide association studies, and polymorphisms in some of these genes influence the risk for alcohol dependence [64]. One unexpected area that has emerged identified genes that regulate immune/inflammatory pathways. Expression of these genes and their regulatory microRNAs are altered in brains from both human alcoholics and mice treated with alcohol [65]. Also, drugs that target immune signaling decrease alcohol consumption in rodent models [66]. These findings point to the possibility of unexplored, non-traditional targets for medication development to reduce alcohol consumption and prevent relapse. The future of alcohol research will rely on the use of multi-faceted approaches to first identify key targets and then exploit pharmacotherapies to reduce aspects of human alcohol dependence such as withdrawal signs, craving, and relapse.

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Highlights

- Structural, computer modeling, and mutagenesis studies reveal direct sites for alcohol on LGICs
- Alcohol alters ion channel function indirectly via receptor phosphorylation and trafficking
- Mutant mice and genetic association studies demonstrate role of LGICs in alcohol dependence
- Combining structural, functional, behavioral, and genomic approaches is crucial for identifying sites of alcohol action

Figure 1. X-ray structures of wild-type and ethanol-sensitized GLIC

(a) Wild-type GLIC (PDB ID 4HFI), colored by residue B-factor according to scale at bottom. For clarity, two proximal subunits are hidden, revealing three distal subunits surrounding channel pore. Lower panel shows inter-subunit transmembrane cavity (light grey surface) formed by the extracellular portions of M1–M2 from one subunit and M2–M3 from the neighboring subunit, and occluded on one end by the F140 side chain (dark grey spheres). (b) Ethanol-sensitized GLIC variant (F140A) (PDB ID 4HFB), depicted as in panel (a). Lower panel shows expanded inter-subunit cavity containing resolved water (red sphere). (c) GLIC F140A mutant co-crystallized with ethanol (PDB ID 4HFE), depicted as in panel (a). Lower panel shows inter-subunit cavity containing ethanol (orange and red spheres). Figure and legend are from Saguet et al., 2013 [19].

Table 1

Timeline for resolution of LGIC structure and ethanol action.

1993	Cryo-electron microscopy structure (low resolution, 9 Å) of nACh receptor
2001	X-ray structure (high resolution, 2.7 Å) of ACh-binding protein
2002	Transmembrane domain of LGICs predicted to have 4 α -helices per subunit
2003	Cryo-electron microscopy structure (moderate resolution, 4 Å) of nACh receptor
2003-2004	Homology models of LGICs based on multiple templates
2008-2011	X-ray structures of ELIC and GLIC
2011	GLIC mutations render it ethanol sensitive
2011-2013	Molecular dynamics simulations of ethanol interactions with LGICs
2013	X-ray structures of single GLIC in open and closed states with ethanol-sensitive and -resistant models; NMR structure of nACh receptor transmembrane domains

nACh-nicotinic acetylcholine; LGIC-ligand-gated ion channel ; ELIC- *E. chrysanthemi* ligand-gated ion channel; GLIC- *Gloeobacter violaceus* ligand-gated ion channel; NMR-nuclear magnetic resonance.