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Ethanol: Novel Actions on Nerve Cell Physiology Explain Impaired Functions

Esa R. Korpi, Riikka Mäkelä, and Mikko Uusi-Oukari

Molecular biological tools have revealed receptor proteins for excitatory and inhibitory neurotransmitters on cell membranes as targets of ethanol action. Behavioral and pharmacogenetic assays using rodent lines have supported this neurotransmitter theory of ethanol action and given a firm basis for future identification of the relevant genes and the central physiological processes vulnerable to ethanol.

Ethyl alcohol, ethanol, is present endogenously at minor concentrations, but the main interest in this relatively simple molecule is the strong alterations in brain function that ethanol produces when ingested at “pharmacological” doses. Ethanol produces a deep depression of brain cell function with concomitant depression of brain energy metabolism. Novel information of ethanol actions will hopefully help us in understanding and developing treatments for alcohol poisoning and alcohol addiction. These two final goals of ethanol studies illustrate the hierarchical levels of the nervous system processes: alcohol intoxication may be partly explained by direct molecular interactions of ethanol and nervous system structures, whereas the addiction mechanism involves long-term learning processes.

This review focuses on recent experiments that have changed the ideas about ethanol action from a general membrane destabilizer to a more selective effector at specific functional proteins on nerve cell membranes. Furthermore, we present an overview of experiments on current animal models used to reveal the genetic basis for ethanol-associated functional impairments.

The earlier hypothesis on membrane fluidization has been challenged because the molecular ratio of membrane phospholipids to ethanol at intoxicating concentrations is too high and because an increase of one degree in temperature produces a similar alteration in membrane fluidity at an anesthetic concentration of ethanol (60–80 mM). A further direct challenge comes from the observation that a firefly luciferase enzyme is inhibited by general anesthetic agents in direct relation to their lipid solubilities and to their anesthetic potencies in tadpoles, even though the enzyme is lipid free and soluble in cytoplasm (4). The mechanism apparently involves competitive inhibition of the substrate, luciferin, binding on the enzyme. Until now, a luciferase-like enzyme, the blinking of which would be eliminated (“passed out”) by relevant concentrations of ethanol, has not been found in the brain, but an analogous situation may be found in ligand-gated ion channels of nerve cells.

**Targets of ethanol action: proteins over lipids**

The earlier hypothesis on membrane fluidization has been challenged because the molecular ratio of membrane phospholipids to ethanol at intoxicating concentrations is too high and because an increase of one degree in temperature produces a similar alteration in membrane fluidity.
Ethanol potentiation of inhibitory receptor channels

Ethanol stimulates the function of the main inhibitory neurotransmitter receptor, the GABA\(_\alpha\) receptor. This potentiating effect of ethanol has been demonstrated in many electrophysiological and biochemical studies. This specific effect of ethanol may largely explain the depressant action of ethanol on brain cells. GABA\(_\alpha\) receptor anion channels are formed from pentameric complexes of an unknown set of the 14 subunits so far cloned (\(\alpha1\)-6, \(\beta1\)-3, \(\gamma1\)-3, \(\delta\), and \(\epsilon\)). With recombinantly expressed mouse receptors, an alternatively spliced long form of the \(\gamma2\)-subunit (\(\gamma2L\)) was required for ethanol potentiation of GABA\(_\alpha\) responses at low millimolar concentrations (13). The eight additional amino acids in the \(\gamma2L\)-variant form a protein kinase C (PKC) phosphorylation site in the intracellular loop between the third and fourth transmembrane domains. Therefore, this structural requirement suggests either that ethanol might work by altering the phosphorylation/dephosphorylation state of a population of GABA\(_\alpha\) receptors or that the phosphorylation of the \(\gamma2\)-subunit is required for ethanol action.

It should be noted that the ethanol potentiation has not been detected in all experiments, and the electrophysiological work by Criswell et al. (3) with GABA\(_\alpha\) receptor subtype-selective drugs indicates that ethanol sensitivity in native receptors in vivo depends on the \(\alpha1\)-subunit rather than the \(\gamma2L\)-variant. Because the \(\alpha1\)-subunit is the most abundant and widespread in the brain among the GABA\(_\alpha\) receptor subunits, it is possible that the other receptor subtypes are not required at all for the depressant actions of ethanol. This would be consistent with the strong attenuation by ethanol of brain glucose utilization. In agreement, high anesthetic ethanol concentrations have been shown to consistently potentiate GABA\(_\alpha\) agonist responses also on recombinant receptors lacking the \(\gamma2L\)-variant. In conclusion, the function of the GABA\(_\alpha\) receptors may be potentiated by an action on extracellular (\(\alpha1\)) or intracellular (\(\gamma2L\)) receptor domains.

The significance of the NH\(_2\)-terminal extracellular domain in ethanol effects is suggested by a recent study comparing the effects of ethanol on recombinant receptors composed of different \(\alpha\)-subunit variants of the inhibitory glycine receptor (GlyR), which is abundant in the spinal cord neurons. The homomeric \(\alpha1\) GlyR is more sensitive to ethanol than the homomeric \(\alpha2\) GlyR (11). These two subunits show a high degree of amino acid homology (76%), and a point mutation in the amino acid 52 (alanine to serine) of the \(\alpha1\)-subunit has been found that decreases the ethanol sensitivity to the level found in the \(\alpha2\) GlyR. The amino acid 52 in the \(\alpha2\)-subunit is threonine, a conservative substitution of serine present in the \(\alpha1\)-mutant receptor, suggesting that this extracellular amino acid plays an important role in the ethanol sensitivity of GlyR \(\alpha\)-variants.

Ethanol inhibition of NM2A type of excitatory glutamate-gated receptor channels

Glutamate receptors, the main excitatory receptors in the brain, can be divided into N-methyl-D-aspartate (NMDA) and non-NMDA receptors. The NMDA receptors are composed of a ubiquitous NR1 subunit making putatively pentameric complexes with NR2A, B, C, or D subunits. Of the ionophoric glutamate receptors, the most sensitive to ethanol inhibition is the NMDA receptor. Ethanol inhibits NMDA responses in some brain regions (e.g., hippocampus, inferior colliculus), whereas it has no effect in others (lateral septum, caudate). Furthermore, in most brain regions, ethanol inhibits NMDA responses in only a subgroup of neurons, possibly only in the neurons in which the NMDA receptor antagonist ifenprodil is able to block the NMDA responses. The combination of an N2B subunit with an NR1 variant produces receptors sensitive to ifenprodil, which in several studies have been the most sensitive of the NMDA receptor subtypes to inhibition by ethanol. However, ethanol is able to inhibit NMDA responses in vivo only in a subpopulation of ifenprodil-sensitive neurons (14). This heterogeneity might be based on differences, e.g., in NR1 splice variants or in posttranslational modifications of the receptor subtypes.

Distinctive mechanisms of ethanol action in nicotinic and ATP-gated channels

Transient expression studies suggest that nicotine responses are inhibited by ethanol in the homomeric \(\alpha7\)-subunit-containing neuronal nicotinic ACh receptors (nAChR). On the other hand, low millimolar ethanol concentrations potentiate the responses to 5-HT in both native and recombinant 5-HT\(_3\) receptors, another cation channel. The 5-HT\(_3\) receptor is supposed to form homomeric receptors of one known subunit. Because ethanol has opposite effects on homomeric nAChR \(\alpha7\) (inhibitory) or 5-HT\(_3\) (potentiating) receptors, it was interesting that its effect on chimeric receptors constructed by joining the NH\(_2\)-terminal extracellular domain of the nAChR \(\alpha7\)-subunit with the transmembrane and COOH-terminal domains from the 5-HT\(_3\) recep-
tor (15) was inhibition of nicotine responses, similar to that in nAChR α7 receptors. This study again suggests that ethanol produces its effect by interacting with the NH2-terminal extracellular domain of the receptor.

Ethanol also inhibits ATP responses at suramin-sensitive P2X receptors (9), which are ATP-gated ion channels. It increases the concentration giving half-maximal response of the receptor for extracellular ATP, whereas it has no effect on maximal response to ATP. This contrasts with the mechanism of ethanol action in the two other excitatory receptors, the NMDA and nAChR α7 receptors, in which the maximal response is decreased by ethanol without a change in the agonist sensitivity. Thus the mechanism of ATP receptor inhibition by ethanol is different from that of the other excitatory receptors, which is not surprising because the ATP channel subunits have a quite different membrane topography with only two transmembrane domains and with the NH2 terminus located intracellularly.

Figure 1 illustrates the possible molecular sites of ethanol action on neurotransmitter receptors, of which the most likely site is the extracellular domain. The exact site of ethanol action is not known at present for any receptor system. We think that this is due to the low potency of ethanol, which eliminates the use of binding studies to reveal the molecular interactions.

The cut-off effect

When the hydrocarbon chain of a series of homologous alcohols (e.g., n-alcohols) is increased, the potency of an alcohol to modulate ligand-gated ion channels increases with increasing chain length until a point is reached at which the potency attains maximum and then declines or disappears with further increase in chain length. This cut-off effect occurs in the anesthetic and membrane-perturbing potency as the chain length reaches 12 carbons. The cut-off points of n-alcohols have been determined for most ligand-gated ion channels (Table 1). As the membrane-disordering effect of n-alcohols disappears with chain length above 12, no clear conclusion about protein or lipid site of action of alcohols can be made from the cut-off chain lengths of n-alcohols at the inhibitory GABA_A receptors or GlyR (Table 1). In contrast, the cut-off point is reached at shorter chain lengths at the excitatory receptors, suggesting a direct interaction between the n-alcohols and the receptor protein. It is proposed that the receptor contains a hydrophobic pocket where the alcohol molecule binds and exerts its effects on the receptor. When the chain length is increased above the cut-off point, the alcohol molecule is sterically hindered to bind in the pocket and as a result fails to modulate the receptor function (Fig. 2). This kind of size exclusion type of cut-off effect

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** Schematic representation of one subunit of a pentameric ligand-gated ion channel receptor complex. Shaded area indicates membrane-spanning domains of the subunit. Proposed regions affecting ethanol sensitivity/being responsible of ethanol action are numbered. Ethanol may act 1) by changing membrane fluidity (unlikely mechanism) or by binding to a hydrophobic pocket of the subunit protein within the membrane, 2) by interacting directly with the NH2-terminal extracellular domain of the subunits, or 3) on the phosphorylation/dephosphorylation of the intracellular domain of receptor subunits.

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**TABLE 1. Characteristics of ethanol effects on ligand-gated ion channels**

<table>
<thead>
<tr>
<th></th>
<th>Effect of Ethanol on Agonist Responses</th>
<th>Cut-Off Point (chain length)</th>
<th>Subunit Proposed to Confer High Ethanol Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inhibitory</td>
<td></td>
</tr>
<tr>
<td>GABA_A</td>
<td>Potentiation</td>
<td>12–13</td>
<td>y2L</td>
</tr>
<tr>
<td>GlyR</td>
<td>Potentiation</td>
<td>13</td>
<td>α1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Excitatory</td>
<td></td>
</tr>
<tr>
<td>NMDA</td>
<td>Inhibition</td>
<td>9</td>
<td>NR2B</td>
</tr>
<tr>
<td>nAChR</td>
<td>Inhibition</td>
<td>ND</td>
<td>α7</td>
</tr>
<tr>
<td>5-HT3</td>
<td>Potentiation</td>
<td>7</td>
<td>5-HT3</td>
</tr>
<tr>
<td>ATP</td>
<td>Inhibition</td>
<td>4</td>
<td>PX2</td>
</tr>
</tbody>
</table>

GABA_A, γ-aminobutyric acid type A; GlyR, glycine receptor; NMDA, N-methyl-D-aspartate; nAChR, nicotinic acetylcholine receptor; 5-HT, serotonin; ND, not determined for the neuronal α7-subunit.

“The exact site of ethanol action is not known…”
has been clearly demonstrated for the ATP-gated ion channel. n-Alcohols inhibit ATP responses in the potency order of 1-propanol > ethanol > methanol, whereas 1-butanol or alcohols with longer chain have no effect on them (10). The hydrophobic pockets responsible for these cut-off effects in receptor channels still need to be localized, and clarification of their roles in low-dose-ethanol effects requires further research.

Ethanol behaviors: a profile of a compound acting on multiple mechanisms

Ethanol has a broad, dose-dependent pharmacological profile (Fig. 3). It is presently clear that, whereas a common molecular basis for all these actions might still exist, their neuronal and neurochemical mechanisms are different.

Ethanol is not the only drug that causes this kind of pharmacological profile; benzodiazepine and barbiturates, which enhance the inhibitory GABA_\text{A} receptor function via defined molecular interactions, and dissociative anesthetics (such as dizocilpine), which block the excitatory NMDA receptors, produce fairly similar behavioral actions in animals and humans. Furthermore, the redundancy of neuronal mechanisms can be appreciated by the fact that, for example, adrenergic a2-agonists and µ-opioid receptor agonists produce many ethanol-like behavioral actions. Therefore, it is not possible to determine the mechanism of action of ethanol just by comparing its profile with those of psychotropic drugs with more specific targets of action.

Selectively bred rodent lines support a genetic component in ethanol actions

Selective breeding of rodent lines for differential sensitivity to ethanol-related behaviors has been used to establish 1) the genetic basis of ethanol-related behaviors and 2) correlations among behavioral, physiological, and neurochemical phenotypes and genetic factors. During the past three decades, several rodent line pairs have been produced by that method. We discuss here two line pairs, the ANT/AT rat line pair and the long sleep/short sleep (LS/SS) mouse line pair, because they have been the focus of intense investigation. These models were developed in such a way as to avoid differential alcohol metabolism. Thus they should illustrate the mechanisms and sensitivity of alcohol action on nervous function.

ANT (ethanol-sensitive) and AT (ethanol-insensitive) rat lines have been selectively outbred for the differential sensitivity to the motor-impairing effects of acute administration of a moderate dose (2 g/kg) of ethanol to model the mechanisms of drinking until intoxication. The impairment of motor performance by ethanol is measured as the ability of the animals to rapidly adjust their body position to resist sliding down on a rough-surfaced tilting plane, the measurement of which is assumed to have a strong cerebellar component. The rat lines show only minor differences in behaviors induced by higher ethanol doses: the hypnotic effect of ethanol, determined as the duration of loss of righting reflex (LORR), is only slightly longer in the ANT than in the AT rats, and the hypothermic effect of ethanol is not different at all between the lines. The selection has not been specific to ethanol, with the ANT rats being also more sensitive to the motor-impairing effects of benzodiazepine-full agonists, barbiturates, some anesthetics, and neurosteroids. Because the drug-induced motor impairment can be antagonized by the GABA_\text{A} receptor antagonist picrotoxin, the efforts have largely focused on finding genetic differences between the ANT and AT rats in brain GABA_\text{A} receptors.

“Selective breeding. . . the genetic basis of ethanol-related behaviors. . . .”

SELECTIVELY BRED RODENT LINES SUPPORT A GENETIC COMPONENT IN ETHANOL ACTIONS

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FIGURE 2. Cut-off effect illustrated for n-alcohols as steric hindrance of the binding to a hypothetical hydrophobic pocket of ATP-gated PX2 receptors. Methyl and methylene groups of the hydrocarbon chains are depicted by C for simplicity.

FIGURE 3. Schematic illustration of ethanol-induced behaviors as related to the degree of potentiation of \( \gamma \)-aminobutyric acid type A (GABA_\text{A}) receptor function and/or of inhibition of glutamatergic receptors, especially those of the N-methyl-d-aspartate type.

\[ \text{C--C--C--C--OH} \quad \text{Pentanol} \]
\[ \text{C--C--C--C--OH} \quad \text{Butanol} \]
\[ \text{C--C--C--C--OH} \quad \text{Propanol} \]
\[ \text{C--C--C--OH} \quad \text{Ethanol} \]
\[ \text{C--C--OH} \quad \text{Methanol} \]
A consistent, qualitative alteration between the ANT and AT rats has only been found in the cerebellum, and, therefore, it is very unlikely that there would be any significant global changes in the GABA_α receptors of either rat line. The potency of benzodiazepine agonists to displace cerebellar benzodiazepine agonist-insensitive (“diazepam-insensitive”) [³H]Ro-15–4513 binding was about 100 times higher in the ANT rats than in AT rats, which has been found to be based on an arginine-to-glutamine point mutation at the amino acid 100 of the GABA_α receptor α6-subunit (8). When studied in recombinant receptors, the mutation in this normally benzodiazepine agonist-insensitive α6-subunit confers diazepam-mediated potentiation of GABA responses, explaining at least the line difference in benzodiazepine sensitivity. The possible role of this mutation on increased ethanol sensitivity still needs to be clarified, but it seems logical that a genetic abnormality in the cerebellar circuitry would make the ANT animals more susceptible to drugs affecting motor coordination, although other genes and other brain regions affecting ethanol sensitivity of motor function cannot be ruled out.

For instance, brain regional 2-deoxy-D-glucose sensitivity of motor function cannot be ruled out. Evidence for the involvement of GABA_α receptor mechanisms has been emerging also in investigations on another rodent line pair produced by selective breeding. The LS (ethanol-sensitive) mouse line, selected for high sensitivity to LORR induced by a high ethanol dose, is also more sensitive to ethanol-induced hypothermia and to LORR induced by benzodiazepines and barbiturates than the SS (ethanol-insensitive) mouse line. Cerebellar Purkinje cells of the LS mice are much more sensitive to alcohol than those of the SS mice in electrophysiological experiments. In contrast to the ANT rats, no differences have been found between the mouse lines in the affinity or density of various GABA_α receptor ligands in brain membranes (including the α6-subunit-dependent diazepam-insensitive [³H]Ro-15–4513 binding), whereas several differences have been found in functional assays between the mouse lines. In ³⁶Cl⁻ flux assays, muscimol, a GABA_α agonist, is a more potent stimulator of the flux into brain membrane vesicles in the LS mice than SS mice, and this stimulation is enhanced by ethanol, benzodiazepines, and barbiturates to a much greater extent in the LS mice than SS mice. Although the exact molecular basis is still unknown, this difference has been confirmed by expressing receptors in Xenopus oocytes from mRNA isolated from the LS and SS mouse brains, supporting its genetic basis (12).

In summary, although especially the GABA_α receptors are implicated in differential ethanol sensitivities of the rodent lines, the data also indicate the involvement of multiple genes, with each gene having probably small contributions to the whole genetically induced behavioral variation. It is also likely that different ethanol-induced effects on brain physiology are regulated at least partly by different genes. Estimates based on the variation of experimental scores have suggested that the minimum number of genes involved in various behaviors is 7–10.

Quantitative trait loci studies reveal multiple chromosomal regions to be identified

Quantitative trait loci (QTL) analysis of behavioral processes is a promising novel technique based on a set of highly divergent mouse strains, which are phenotyped for various behaviors and drug sensitivities and genotyped for hundreds or thousands of mouse genome markers. Scores for behavioral traits and drug effects are then analyzed for correlations with genome loci markers.

Interesting provisional results have been reported for some ethanol-induced behaviors, which collectively witness to a multitude of genetic factors involved (2). For example, with regard to sensitivity to acute motor-impairing or hypnotic actions of ethanol, significant correlations with 3–19 QTLs on more than 10 different mouse chromosomes have been observed in various studies. Because most of these QTLs are provisional and unconfirmed and their strength is affected by the set of inbred strains used (e.g., whether from LS × SS or from C57BL × DBA mouse line crosses), it is still premature to draw any conclusions on the roles of specific genes in specific ethanol behaviors. This analysis supports the earlier ideas that various ethanol-induced behaviors depend on at least three strong QTLs for each behavior and that the chromosomal sites are likely different for motor-impairing and hypnotic sensitivities in line with the data on ANT/AT and LS/SS line pairs (see above).

The QTL data available to date do not yet prove or disprove the involvement of genes affecting, e.g., the GABAergic system. Buck et al. (1) have localized a significant QTL for acute alcohol withdrawal to a region of the mouse chromosome 11 that harbors the genes for the
GABA$_{\alpha}$ receptor $\alpha_{1-}e$, $\alpha_{6-}$, and $\beta_{2}$-subunits. The sensitivity of the QTL method is well illustrated by the estimation of how much this locus accounts for phenotypic (3%) and genotypic (12%) variance. Now that the mouse genome is becoming more and more precisely known, the research will continue using transgenic and other strategies to directly test the involvement of specific genes in ethanol actions.

**Confirmation with specific “knockout” mouse models**

Inactivation of genes of potential interest for ethanol-induced behaviors can be accomplished by homologous recombination with inactivated alleles into mouse germline genome and subsequent production of homozygous mice. To preliminarily test the ANT mutation, the whole GABA$_{\alpha}$ receptor $\alpha_{6}$-subunit has been inactivated and the success of the gene knockout evidenced by the disappearance of the diazepam-insensitive $[{\text{3H}}]$Ro-15–4513 binding from the cerebellar granule cell layer (6, 7). Unexpectedly, the mutant animals also lack most of the cerebellar GABA$_{\alpha}$ receptor $\delta$-subunits, which means that they actually have a double subunit knockout with drastically reduced amounts of high-affinity sites for GABA$_{\alpha}$ agonist muscimol. The $\alpha_{6}^{-/-}$ mutants show normal locomotor activity, and their responses to high ethanol and anesthetic doses are similar to wild-type $\alpha_{6}^{+/+}$ animals (6). Low-dose actions of ethanol and benzodiazepines have not been studied so far, and, for the conclusive experiment, the wild-type allele should be replaced by the ANT mutant subunit because then the animals would have the same receptor levels and normal $\delta$-subunit expression, which would better mimic the genetic situation of the ANT rats.

Site-directed mutagenesis of the cytoplasmic loop of the GABA$_{\alpha}$ receptor $\gamma_{2L}$-subunit suggested a critical role for its phosphorylation by PKC on ethanol potentiation of GABA responses. Therefore, PKC might be genetically variable to explain the LS/SS mouse line difference in hypnotic sensitivity to ethanol. This hypothesis has been supported by the reduced sensitivity of PKC$\gamma$ knockout mice to anesthetic and hypnotic actions of ethanol.

The main problem with the knockout strategy is possible secondary effects on other mechanisms due to plasticity of the developing nervous system, which could be avoided with conditional knockouts or with antisense oligonucleotide strategy in mature animals. As the techniques advance, we will have the possibility to engineer precise alterations in putative sites of ethanol action, as illustrated with recombinant receptors, but even then we face the problem of redundancy of brain mechanisms. Presently known genetic correlates for human alcoholism (or absolutism) involve alcohol metabolism (aldehyde dehydrogenase defects) rather than central nervous system mechanisms. For these reasons, we still need to use other strategies, like the QTL mapping, to find the arrays of genes involved. Then the marker loci can be used to predict the homologous human chromosomal regions to be screened in patient populations.

**References**


Although there have been great strides in the development of antihypertensive drugs, hypertension continues to be a major health problem affecting over 20% of the adult population. If left untreated, hypertension causes damage to the vascular endothelium, resulting in a proliferative response, arteriosclerosis, and consequent end-organ damage leading to increased risks of stroke, coronary arterial disease, myocardial infarction, congestive heart failure, and chronic renal failure (10). Although hypertension is often regarded as the cause of renal disease and chronic renal failure, it is also recognized that hypertension may be the consequence of defects in renal microcirculatory and/or tubular transport function that compromise the normal capability of the kidney to maintain sodium balance at normal arterial pressures. Even when there is no primary intrarenal derangement, an impairment in renal function secondary to inappropriate humoral or neural stimulation to the kidney may exist. In essence, a widely held premise is that hypertension cannot coexist in the presence of normal kidney function (3, 6, 9).

Responses to unilateral renal arterial stenosis

One very intriguing experimental model of hypertension is the two-kidney, one-clip (2K1C) Goldblatt model (4) in which hypertension is induced by unilateral stenosis of the renal artery (2, 7, 8, 11). The clip is not severe enough to cause ischemia; however, the reduced renal perfusion pressure stimulates increased renin synthesis and release from the clipped kidney. As shown in Fig. 1, renin enzymatically cleaves angiotensin I (ANG I) from angiotensinogen, and angiotensin-converting enzyme (ACE) acts on ANG I to produce angiotensin II (ANG II). Circulating ANG II, via its direct vascular effects, acutely increases total peripheral resistance and