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Ethanol and the benzodiazepine-GABA receptor-ionophore complex

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Summary. Ethanol has a pharmacological profile similar to that of classes of drugs like benzodiazepines and barbiturates, which enhance GABAergic transmission in the mammalian CNS. Several lines of behavioral, electrophysiological and biochemical studies suggest that ethanol may bring about most of its effects by enhancing GABAergic transmission. Recently, ethanol at relevant pharmacological concentrations has been shown to enhance GABA-induced ^{36}Cl-fluxes in cultured spinal cord neurons, synaptoneurosomes and microsacs. These enhancing effects of ethanol were blocked by GABA antagonists. Ro15-4513, an azido analogue of classical BZ antagonist Ro15-1788, reversed most of the behavioral effects of ethanol and other effects involving ^{36}Cl-flux studies. The studies summarized below indicate that most of the pharmacological effects of ethanol can be related to its effects on GABAergic transmission.
Key words. Ethanol; GABA receptor complex; chloride channels; Ro15-4513.

The molecular mechanism by which ethanol produces its effects, and the neuronal components involved in ethanol actions, have been a matter of debate over the years. A variety of neuronal pathways have been implicated in various states of alcoholism[48]. However, recent evidence indicates that drugs which have a pharmacological profile similar to ethanol, i.e. benzodiazepines (BZs) and barbiturates, appear to bring about their pharmacological effects by facilitation of inhibitory transmission mediated by GABA. Based on several lines of behavioral, electrophysiological and radioligand-binding studies, and functional assays, it is becoming apparent that ethanol may also mediate many of its effects via GABAergic transmission[17, 53, 58]. This paper will summarize the evidence which implicates $GABA_A$ receptor system in the pharmacological effects of ethanol.

GABA-BZ receptor-ionophore complex

GABA receptor is an oligomeric complex, composed of four binding sites which bear an allosteric relationship to each other (fig. 1). Radioligand binding studies have shown that these sites include GABA recognition sites, BZ sites and picrotoxin sites[38, 51]. Although previous studies suggested that barbiturates act at the picrotoxin sites[56], recent studies based on equilibrium binding and dissociation kinetic studies with [^{35}S]t-butylbicyclophosphorothionate (TBPS), a ligand which binds to the picrotoxin site[39, 45], have shown that barbiturate sites are distinct from the picrotoxin sites[28, 57, 59].

GABA receptor activation opens chloride channels, resulting in hyperpolarizing responses[4, 6]. GABA-mediated responses are blocked by bicuculline, picrotoxin and

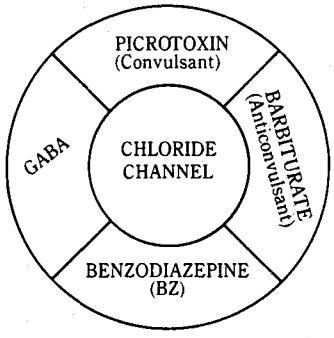

Figure 1. Potential sites of drug action at the GABA-benzodiazepine receptor-ionophore complex[57].

pentylenetetrazol and facilitated by BZ agonists and depressant/anticonvulsant barbiturates[4,6,15,44]. Furthermore, β-carbolines (agonists/antagonists/inverse agonists) modify GABA response in accordance with their pharmacological activity[7]. The GABA receptor has recently been cloned and is believed to be composed of two α (BZ-receptor) and two β (GABA-receptor) subunits[43]. However, the exact subunit composition and the stoichiometry of GABA receptor remains to be resolved.

Ethanol and GABA receptor complex

Electrophysiological studies

The ability of ethanol to potentiate GABA-mediated responses in electrophysiological experiments has been controversial. Several investigators have shown that ethanol facilitates GABA-mediated responses in preparations like cat spinal cord[3], frog spinal cord[9], cortical neurons[35] and substantia nigra[33], and brings about recurrent inhibition of the dentate gyrus of the hippocampus[16]. However, negative results have also been reported[19].

Behavioral studies

Several lines of behavioral studies using diverse paradigms like ethanol-induced motor incoordination, loss of righting reflex, the anticonvulsant effect of ethanol, and ethanol-induced withdrawal symptoms, have also implicated GABA synapses in the actions of ethanol. Thus, the GABA antagonist bicuculline attenuates ethanol-induced motor impairment[14]. Further, agents which elevate GABA levels in the CNS were reported to enhance ethanol-induced motor incoordination[11,12,21]. Drugs which bind to the picrotoxin site of the GABA receptor complex, like picrotoxin and isopropylbicyclophosphate, decrease ethanol-induced loss of the righting reflex[32]. Our laboratory has previously demonstrated that ethanol exhibited an anticonvulsant effect against bicuculline, picrotoxin and strychnine[41]. More importantly, we also observed that subeffective doses of ethanol, in combination with subeffective doses of other facilitators of GABAergic transmission, potentiated each others' effects in vivo[41]. Similar results were also observed against electroshock-induced seizures[40]. Others have also reported an anticonflict effect of ethanol, and an anticonvulsant effect against chemoconvulsants[21,22]. Frye and co-workers[12] have implicated the GABA system in audiogenic seizures associated with ethanol withdrawal. These observations, coupled with the fact that some of the ethanol withdrawal symptoms can be prevented by GABA mimetics, indicates the involvement of the GABAergic system in the pharmacological effects of ethanol, and in part, in ethanol withdrawal.

Radioligand binding studies

Effects of ethanol on the binding to various sites on the GABA receptor complex have been investigated both in vitro and following acute and/or chronic administration of ethanol. In vitro, using membrane homogenates, it was found that ethanol does not affect the binding of GABA and BZ agonists to their binding sites[13]. However, ethanol was reported to enhance the binding of [^{3}H]diazepam to a lubrol-solubilized fraction in vitro[8]. The reason for this discrepancy is not clear and it is possible that the effect observed in a detergent-solubilized preparation could be nonspecific or artifactual, since no reproducible effect could be observed in membrane homogenates[8]. Ethanol does inhibit the binding of [^{35}S]t-butylbicyclophosphorothionate (TBPS) to the picrotoxin site in vitro[23,49]. This interaction of ethanol with TBPS binding sites appears to be allosteric, since it accelerates the dissociation kinetics of TBPS[29]. Since TBPS binding is also inhibited by barbiturates, it was suggested that ethanol may modulate GABAergic transmission via the picrotoxin site[29,49]. However, the concentrations of ethanol that inhibited TBPS binding (EC_{50} ~ 500 mM) are lethal. The importance of ethanol inhibition of TBPS binding is not clear, but it could be involved in ethanol intoxication.

Chronic ethanol administration does not alter the binding characteristics of TBPS or BZ agonists to their receptor sites[23,42,49]. Likewise, the ability of GABA to inhibit TBPS binding (IC_{50}) or to enhance BZ binding (EC_{50}) were also not altered following chronic ethanol administration or during withdrawal[42]. However, complex results are observed on GABA receptor binding following chronic ethanol administration[50,52,60]. Based on these studies, it was suggested by this laboratory that the effects of ethanol on GABAergic transmission may occur at the level of coupled chloride channels[42,49]. Recent investigations described below, using functional studies, have supported this contention.

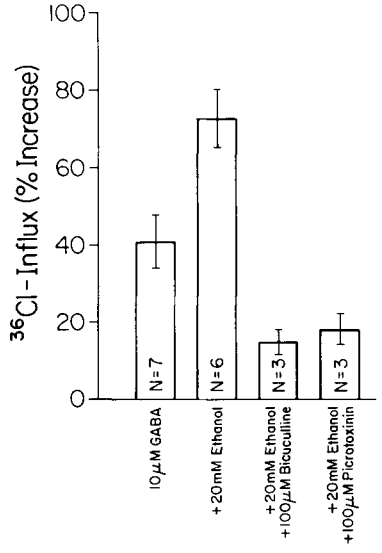

Figure 2. Ethanol (20 mM) enhancement of GABA (10 μM)-induced ^{36}Cl-influx and its antagonism by bicuculline and picrotoxinin in spinal cord neurons. Ethanol (up to 20 mM) does not alter the basal ^{36}Cl-influx in these neurons[31].

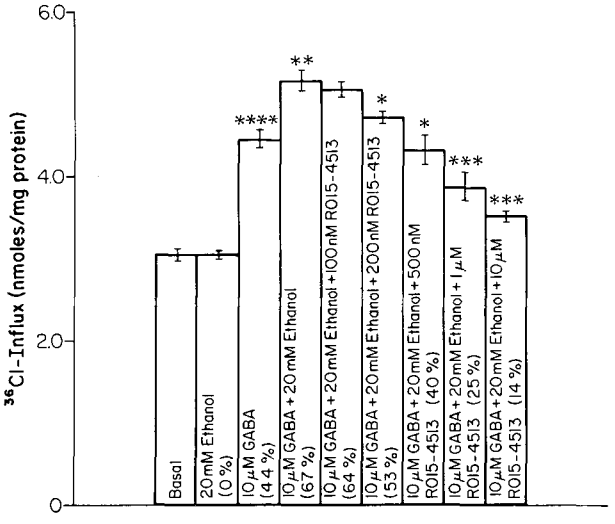

Figure 3. Ability of Ro15-4513 to reverse ethanol enhancement of GABA-induced ^{36}Cl-influx in spinal cord neurons[31].

Effect of ethanol on a functional assay using GABA-induced ^{36}Cl-flux

To demonstrate that ethanol does, in fact, modulate GABAergic transmission, recent studies have utilized functional assays using GABA-mediated ^{36}Cl-fluxes in vitro. Ethanol has been reported to potentiate $GABA_A$ receptor mediated ^{36}Cl-fluxes in cultured spinal cord neurons[31,54], neurosynaptosomes[47] and microsacs[1]. Ethanol at pharmacologically relevant concentrations potentiates $GABA_A$ agonist-stimulated ^{36}Cl-flux. Allan and Harris[1] reported that ethanol, while potentiating muscimol-stimulated ^{36}Cl-flux in the cerebellum of long sleep (LS) mice did not do so in the cerebellum of mice of the short sleep (SS) strain, suggesting a differential sensitivity to ethanol in the two strains. Additionally, these investigators did not observe a potentiating effect of ethanol in hippocampal preparations from LS or SS mice. It was also reported that LS mice exhibited tolerance to the ethanol potentiating effect following acute and chronic ethanol treatments[2]. In contrast, Suzdak et al.[47] observed that ethanol (20–50 mM), besides potentiating the effect of muscimol, also directly stimulated ^{36}Cl-flux. The effect of ethanol was biphasic, and was blocked by GABA antagonists. These investigators reported that potentiation of muscimol-stimulated ^{36}Cl uptake was a result of an increase in the V_{max}, rather than a change in the K_m value of muscimol[47].

Over the years, our laboratory has developed cultured spinal cord neurons in order to study GABA synaptic pharmacology. Spinal cord cultured neurons contain specific BZ receptors that are coupled to GABA, barbiturate and picrotoxin sites[20,30,31]. Furthermore, $GABA_A$ agonists produce a concentration-dependent increase in ^{36}Cl-influx, a response which is blocked by bicuculline and picrotoxinin and potentiated by BZ agonists[20,31,54]. Ethanol (5–100 mM) potentiated the effect of submaximal concentrations of GABA (10 μM), with the maximal effect occurring at 50 mM ethanol. Figure 2 shows that a concentration of ethanol (20 mM) which does not alter the basal ^{36}Cl-influx potentiated the effect of GABA on the ^{36}Cl-influx in these neurons. This effect of ethanol was blocked by bicuculline and picrotoxin, and was due to a decrease in the K_m value of GABA from 11.4 ± 0.8 μM to 4.8 ± 0.5 μM[31]. The potentiating effect of ethanol was specific for GABA-gated Cl^- channels, since ethanol did not modify a glycine-induced ^{36}Cl-influx[31]. The potentiating effect of ethanol was also blocked by inverse agonists of the BZ receptors. This effect is similar to that observed with benzodiazepine agonists (unpublished observation). Besides potentiating the effect of GABA ethanol at ≧ 20 mM directly activated Cl^- channels in spinal cord neurons[31]. The direct effect of ethanol was also blocked by GABA antagonists and inverse agonists of BZ receptors. Furthermore, the direct effect of ethanol could not be observed when all the GABA receptors were saturated with the agonist. These results suggest that the direct effect of ethanol is also mediated by GABA-gated Cl^- channels. These results are similar to that reported by Suzdak et al.[47], but differ from Allan and Harris[1], who did not observe a direct effect.

Ro15-4513: An ethanol antagonist

Recently, a great deal of attention has focused on the observation that an azido analogue of the BZ receptor

antagonist Ro15-1788, Ro15-4513, could block many of the effects of ethanol, including effects on GABA receptor-gated Cl^- channels[5,24–27,46]. Effects of Ro15-4513 on ethanol and the GABAergic system have recently been reviewed[58]. Ro15-4513 reverses the behavioral effects of ethanol, which were reversed by the BZ antagonist Ro15-1788[46]. Ro15-4513 has also been reported to reverse intoxication due to large doses of ethanol[10], but others have failed to detect such a protective effect[37]. However, these investigators used massive doses (7.5–15 g/kg) of ethanol. It is feasible that such high doses of ethanol affect a variety of biological processes in addition to GABAergic transmission. Thus, the inability of Ro15-4513 to block lethal effects of ethanol may not be surprising. The concensus seems to be that Ro15-4513 reverses most of the effects of ethanol which may be related to the GABA system, except hypothermia. This is again not surprising, since ethanol-induced hypothermia is not mediated via GABAergic transmission. It may be noted that Ro15-4513 is a partial inverse agonist and also exhibits proconvulsant effects[5,18,36]. While some investigators report selectivity of ethanol antagonistic activity to Ro15-4513[47], others report similar results against pentobarbital and BZ agonists[36].

We have recently observed that Ro15-4513 reversed the inhibition by ethanol of convulsions and mortality due to bicuculline better than those due to picrotoxin, and FG-7142 was less effective. Further, Ro15-4513 was not as effective against the inhibition by pentobarbital of convulsions due to bicuculline and picrotoxin[18]. It is possible that these differences could be due to different experimental paradigms used, species and/or doses of ethanol used in such studies. Ro15-4513 also blocks the ability of ethanol to potentiate GABA-induced ^{36}Cl-fluxes in synaptoneurosomes and spinal cord neurons[31,45,46]. Figure 3 shows that Ro15-4513 blocked the ethanol potentiating effect on GABA induced ^{36}Cl-influx in cultured spinal cord neurons in a concentration-dependent manner. While the effect of Ro15-4513 was reported to be unique in synaptoneurosomes, several other inverse agonists like FG-7142 and DMCM (methyl 6,-7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) reversed the effects of ethanol in spinal cord neurons[31]. Apparently, this effect occurred at concentrations at which Ro15-4513 and FG-7142 did not exhibit inverse agonistic activity in a ^{36}Cl-flux assay[31]. A recent study from our laboratory has also shown that chronic ethanol treatment (intragastric method) produced a selective increase in the number of binding sites for [^{3}H]Ro15-4513 in cortex and cerebellum, but not in hippocampus or striatum[34]. We also reported that ethanol does not interact with [^{3}H]Ro15-4513 binding sites in membrane homogenates. These results are intriguing and suggest that the ability of ethanol to increase Ro15-4513 binding sites following chronic treatment may result by acting at a certain unique lipoprotein domain in close proximity to the Cl^- channels. Furthermore, since chronic ethanol treatment does not alter the BZ agonist binding[42], it is reasonable to speculate that the binding characteristics of BZ agonists/antagonists and inverse agonists like Ro15-4513 may not be identical. This may explain why Ro15-4513, but not BZ antagonist Ro15-1788, blocks the effects of ethanol.

In summary, many of the pharmacological effects of ethanol can be related to its ability to facilitate $GABA_A$ergic transmission in the CNS. This is based on behavioral and electrophysiological studies and on recent biochemical assays using GABA-induced ^{36}Cl flux studies in several preparations. Additional support is provided by the findings that besides GABA antagonists, an inverse agonist of the BZ receptor, Ro15-4513, exhibits a degree of selectivity in blocking the effects of ethanol. In vitro binding studies rule out the direct involvement of GABA, BZ or picrotoxin sites in ethanol-induced tolerance and withdrawal. However, there may be a role for Ro15-4513 binding sites in the action of ethanol, in tolerance to ethanol and in the effects of ethanol withdrawal, besides the coupling mechanism associated with GABA receptor-gated Cl^- channels.

Abbreviations used: GABA = γ-aminobutyric acid; BZ = benzodiazepine(s); TBPS = t-butylbicyclophosphorothionate; Ro15-4513 = (ethyl-8-azido-5, 6-dihydro-5-methyl-6-Oxo-4H-imidazo [1,5α], [1,4] benzodiazepine-3-carboxylate); Ro15-1788 = (ethyl-8-fluoro-5, 6-dihydro-5-methyl-6-Oxo-4H-imidazo [1,5α], [1,4] benzodiazepine-3-carboxylate); FG-7142 = (N-methyl-β-carboline-3-carboxamide).

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Ethanol and opioid receptor signalling

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Summary. Ethanol may modulate endogenous opioid systems by disrupting opioid receptor signalling. Low concentrations of ethanol slightly potentiate μ-opioid receptor binding by increasing receptor B_{max}, and, in some cases, chronic ethanol exposure decreases the density or affinity of the μ-opioid receptors. By contrast, high concentrations of ethanol acutely decrease δ-opioid receptor binding by decreasing receptor affinity, whereas chronic exposure of animals and neuronal cell lines to lower concentrations of ethanol leads to possibly adaptive increases in the density or affinity of the δ-opioid receptors. In the neuronal cell line NG108-15, ethanol does not up-regulate the δ-opioid receptor by blocking receptor degradation or endocytosis, but protein synthesis is required for this response. Up-regulation of the δ-opioid receptor renders ethanol-treated NG108-15 cells 3.5-fold more sensitive to opioid inhibition of adenylyl cyclase. Long-term treatment with ethanol also increases maximal opioid inhibition in NG108-15 cells, possibly by decreasing levels of $G\alpha_s$ and its mRNA. Ethanol differentially modulates signal transduction proteins in three additional neuronal cell lines, N18TG2, N4TG1, and N1E-115. Ethanol-treated N18TG2 cells show the least up-regulation of the δ-opioid receptor, little heterologous desensitization of adenylyl cyclase, and no changes in $G\alpha_s$ or $G\alpha_i$. By contrast, ethanol-treated N1E-115 cells show the largest up-regulation of the δ-opioid receptor, the most heterologous desensitization of adenylyl cyclase, and concentration-dependent decreases in $G\alpha_s$ and increases in $G\alpha_i$. Further analysis of these related neuronal cell lines may help to identify the molecular elements that endow some, but not all, neuronal cells with the capacity to adapt to ethanol.
Key words. Endogenous opioid systems; ethanol; Gα-proteins; receptor signalling; up-regulation; μ-opioid receptor; δ-opioid receptor.

Alcohol and endogenous opioid systems

There is considerable evidence that ethanol interacts with endogenous opioid systems to produce some of its central nervous system (CNS) effects[4, 5, 81]. ICI 174864, a selective δ-opioid receptor antagonist, can block ethanol-induced hypothermia and sedation when microinjected into discrete brain regions[81]. Moreover, the opiate antagonist naloxone can attenuate the ethanol withdrawal syndrome when given during and after the administration of ethanol[3, 4]. Heritable differences in susceptibility to alcoholism may also be related to an ethanol-opioid interaction. In inbred strains of mice, ethanol consumption correlates inversely with brain levels of [Met]enkephalin[5].

Ethanol could modulate the activity of endogenous opioid systems through effects on the synthesis, processing, or release of opioid peptides, or on opioid receptor signalling (fig. 1). Effects of ethanol on the biosynthesis and regulation of opioid peptides are described elsewhere in this volume[18]. Here, I will review the effects of ethanol on opioid receptor signalling in brain and in neuronal cells.

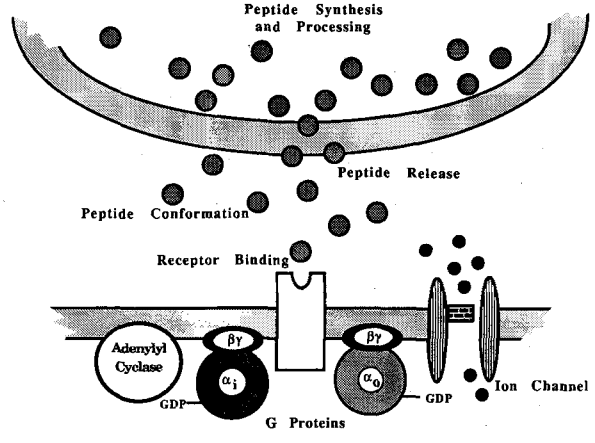

Figure 1. Possible sites of action of ethanol on endogenous opioid systems. Ethanol could alter the synthesis, release, conformation, or receptor binding of opioid peptides, or the events that follow receptor activation. Opioid receptors couple with G proteins to regulate adenylyl cyclase or ion channels and ethanol could disrupt opioid signalling at any of these loci.