Alcohol-sensitive GABA receptors and alcohol antagonists

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Alcohol is one of the oldest and most widely used and abused of all psychoactive drugs. Although alcohol ingestion impacts most organ systems, its effects on the brain are of considerable interest, given alcohol's many neuropharmacological actions, including its intoxicating, sedative, anxiolytic, reinforcing, and addictive properties (1). However, elucidating the cellular and molecular targets for alcohol's important pharmacological actions has proven challenging. Alcohol has a relatively simple chemical structure, it has pleiotropic effects in disordering membrane lipids and proteins, and relatively high clinically relevant (5–30 mM) tissue concentrations are required for its actions. Thus, it is unlikely that any single molecular mechanism (or target for that matter) will explain all of the relevant pharmacology of this important drug. Despite these caveats, research over the past two decades has identified a number of potential alcohol targets in brain, including various G protein-coupled receptors and ligand-gated ion channels (1). In some cases, alcohol has been shown to modify these targets at pharmacologically relevant concentrations; however, with few exceptions, the concentrations (or doses) of alcohol studied have been well above those that cause acute intoxication in animals, including humans. Despite considerable work in this area, two important questions remain largely unanswered. Can any of the clinically relevant neuropharmacological actions of alcohol be attributed to a direct interaction with one or more "specific" protein targets, such as a receptor or ion channel? If so, can the neuropharmacological actions of alcohol be mimicked, modified, or even blocked by a much more specific drug acting at this same target(s)? In this issue of PNAS, Hanchar *et al.* (2) and Wallner *et al.* (3) provide compelling data for a rather specific and pharmacologically relevant alcohol binding site on a subtype of $GABA_A$ receptor composed of $\alpha4/6\beta3\delta$ subunits. Remarkably, they show that the behavioral alcohol antagonist Ro15–4513 binds to the same (or overlapping) site and competes with and antagonizes the actions of low to moderate concentrations of alcohol in potentiating GABA-induced Cl^- currents. Thus, the authors provide exciting new evidence for a highly specific interaction of alcohol with a subtype of $GABA_A$ receptor that may mediate (at least in part) some of this drug's most important behavioral effects.

Alcohol and GABA

GABAergic neurotransmission and $GABA_A$ receptors in particular have long been implicated in mediating at least some of the pharmacological actions of alcohol (1). $GABA_A$ receptors are also the molecular targets for benzodiazepines and anesthetic barbiturates (4), both of which share neuropharmacological properties and show cross-tolerance and cross-dependence with alcohol (1). Despite much circumstantial *in vivo* evidence, demonstrating consistent direct effects of alcohol on $GABA_A$ receptors, especially at clinically relevant concentrations, has proven problematic (5). In the mid-1980s, several laboratories including my own (6, 7), using an *in vitro* biochemical assay that measures $GABA_A$ receptor-mediated $^{36}Cl^-$ flux in synaptoneurosomes, showed that alcohol potentiates $GABA_A$ receptor activity at low (<20 mM) intoxicating concentrations (6). We also found that a novel imidazobenzodiazepine, Ro15–4513, completely blocked the ability of alcohol (but not pentobarbital) to enhance $GABA_A$ receptor-mediated $^{36}Cl^-$ flux (8). Our work on this compound was prompted by earlier work by scientists at Roche demonstrating that Ro15–4513 had unique "anti-alcohol" properties *in vivo* (9, 10). We extended these findings and also observed that Ro15–4513 possessed potent anti-alcohol actions *in vivo* that could be differentiated from other benzodiazepine receptor antagonists and inverse agonists (11). In fact, in our hands, benzodiazepine receptor antagonists and most inverse agonists actually blocked the anti-alcohol actions of Ro15–4513 (11). However, it was also clear from these early studies that Ro15–4513 blocks only some of the behavioral effects of low to moderate doses of ethanol (8–12) and that its intrinsic inverse agonist properties could confound the interpretation of the behavioral data (11, 12). Consequently, these findings were met with considerable skepticism, and at least two controversies emerged. First, did alcohol bind directly to $GABA_A$ receptors, or did it somehow potentiate GABA indirectly? Although several groups observed similar effects of alcohol on $GABA_A$ receptor activity measured using $^{36}Cl^-$ flux in synaptoneurosomes (7, 13), many, if not most, electrophysiological studies simply failed to find direct alcohol-induced augmentation of GABA-mediated synaptic events (5, 14). Second, was the imidazobenzodiazepine "alcohol antagonist" Ro15–4513 really a "selective" alcohol antagonist, or did it merely reverse some of the biochemical, electrophysiological, and behavioral effects of alcohol by virtue of its inverse agonist properties (15, 16), i.e., by simply producing the opposite effects of alcohol? As to the former, little was known in those days of the rather remarkable heterogeneity of $GABA_A$ receptor subunits, their assembly into functional heteropentameric $GABA_A$ receptors, and the pharmacological implications of this receptor subunit heterogeneity (17). In this regard, several laboratories have recently reported a critical role for the δ subunit (expressed together with $\alpha4$ or $\alpha6$ and $\beta3$ subunits) in conferring heightened sensitivity to alcohol on $GABA_A$ receptors. Indeed, the work of Hanchar *et al.* (2) and Wallner *et al.* (3) builds on earlier work by their laboratory (18) and others (19, 20) demonstrating that low concentrations of alcohol augment GABA acting at δ subunit-containing $GABA_A$ receptors. Significantly, these $GABA_A$ receptors have been shown by several laboratories to be extrasynaptic in location, meaning that, in contrast to synaptic $GABA_A$ receptors, they are activated in a "tonic" as opposed to "phasic" manner by very low extrasynaptic concentrations of GABA (21). Parenthetically, one might postulate that the synaptoneurosomes that we and others used to measure alcohol's effects on $GABA_A$ receptor-mediated $^{36}Cl^-$ flux (6, 7, 13) are enriched in these extrasynaptic GABA receptors.

Alcohol and Ro15–4513-Sensitive $GABA_A$ Receptors

Given the marked sensitivity of extrasynaptic $\alpha4/6\beta3\delta$ $GABA_A$ receptors to alcohol (18), Hanchar *et al.* (2) first studied the binding of [^{3}H]Ro15–4513 to both native and recombinant $GABA_A$ receptors containing $\alpha4/6\beta3\delta$ subunits. Previous work had suggested that benzodiazepine binding to $GABA_A$ receptors required γ

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subunits, so it was quite surprising that Ro15–4513 (and the structurally related benzodiazepine antagonist Ro15–1788) bound with such high affinity to $\alpha4/6\beta3\delta$ $GABA_A$ receptors ($K_d \approx 7$ nM). Next, the authors tested whether ethanol itself inhibits [^{3}H]Ro15–4513 binding to δ subunit-containing native and recombinant $GABA_A$ receptors. Remarkably, ethanol inhibited [^{3}H]Ro15–4513 binding to δ subunit-containing receptors with an IC_{50} of ≈12 mM but was without effect on [^{3}H]Ro15–4513 binding to $\alpha4\beta3\gamma2$ GABA receptors. Significantly, ethanol completely inhibited [^{3}H]Ro15–4513 binding to $\alpha4\beta3\delta$ GABA receptors, and subsequent kinetic analysis using saturating and nonsaturating concentrations of [^{3}H]Ro15–4513 and ethanol, respectively, strongly suggests a direct competitive (as opposed to allosteric) binding interaction between the two molecules. Finally, a series of benzodiazepine and β-carboline receptor ligands were tested as inhibitors of [^{3}H]Ro15–4513 binding to $\alpha4/6\beta3\delta$ GABA receptors. Only those compounds [FG7142, Ro15–1788, β-carboline-3-carboxyethyl ester (β-CCE)] previously reported to block the anti-alcohol effects of Ro15–4513 *in vivo* (11) inhibited [^{3}H]Ro15–4513 binding to $\alpha4/6\beta3\delta$ $GABA_A$ receptors.

In their companion paper, Wallner *et al.* (3) extend these intriguing findings by showing that the enhancement of $GABA_A$ receptor-mediated Cl^- currents (measured in $\alpha4/6\beta3\delta$ $GABA_A$ receptors expressed recombinantly in *Xenopus* oocytes) by ethanol (30 mM) was completely reversed by Ro15–4513 (300 nM). Moreover, Ro15–4513 did not reduce "basal" GABA-induced currents mediated by $\alpha4/6\beta3\delta$ $GABA_A$ receptors and thus did not behave as an inverse agonist at concentrations that blocked ethanol. The IC_{50} for Ro15–4513's actions in blocking ethanol's GABA-enhancing effects on $\alpha4\beta3\delta$ $GABA_A$ receptors was ≈10 nM, very similar to its K_d for binding to these same receptors (2). At higher alcohol concentrations (>100 mM), a proportion of the ethanol-induced enhancement was not blocked, even by high concentrations of Ro15–4513, and therefore, $\alpha4/6\beta3\delta$ $GABA_A$ receptors appear to respond to high alcohol concentrations in a Ro15–4513-insensitive manner. The latter was abolished in recombinantly expressed receptors where the $\beta3$ wild-type subunit was replaced with a mutated $\beta3$N265M subunit, resulting in only Ro15–4513-sensitive alcohol enhancement. Finally, both β-carboline inverse agonists, β-CCE and FG7142 (but not the potent inverse agonist DMCM), as well as Ro15–1788 were shown to reverse the inhibition by Ro15–4513 of alcohol-induced enhancement of δ subunit-containing GABA receptors, results that are strikingly reminiscent of the earlier biochemical and behavioral work (8, 11). Of note, β-CCE actually potentiated the effects of low concentrations of ethanol (3 mM) on $\alpha4\beta3\delta$ $GABA_A$ receptors and even stimulated these receptors in the absence of alcohol (3).

Taken together, these findings suggest that a subtype of extrasynaptic $GABA_A$ receptor that contains δ subunits and gives rise to tonic (sustained) GABAergic inhibition in brain (21) is indeed an important molecular target for alcohol, especially at alcohol concentrations achieved during social alcohol ingestion. Moreover, the data suggest the presence of a rather "specific" alcohol binding site on these same $GABA_A$ receptors that is shared by the behavioral alcohol antagonist Ro15–4513. If correct, these findings help clarify 20 years of puzzling and often contradictory findings on alcohol, $GABA_A$ receptors, and the alcohol antagonist Ro15–4513. Nonetheless, as the authors themselves point out, these controversies are unlikely to be resolved soon because a recent report by Borghese *et al.* (22) claims that δ subunit-expressing $GABA_A$ receptors do not respond to alcohol, a discrepancy that hopefully can be resolved.

There are several obvious implications and questions that derive from these exciting findings. What other pharmacological effects of alcohol might be mediated by these alcohol and Ro15–4513-sensitive extrasynaptic $GABA_A$ receptors? Could the reinforcing or addictive properties of alcohol be mediated by these receptors? Could highly specific drugs be developed (hopefully devoid of other undesirable intrinsic properties) that would selectively block or even mimic alcohol's effects on these $GABA_A$ receptors? In this regard, it is tempting to speculate that the neuroactive steroids (allotetrahydroDOC or allopregnanolone) (23, 24), which have been shown recently to augment δ subunit-containing $GABA_A$ receptors (25), may represent endogenous "alcohol-like" agonists at these same receptors. Do naturally occurring δ (or related) $GABA_A$ receptor subunit polymorphisms exist in animals, including humans, and do they alter alcohol-related behaviors? Finally, similar pharmacologically relevant alcohol-binding sites undoubtedly exist on other important neuronal proteins. Are there common structural motifs for these alcohol-binding sites like that recently revealed by the high-resolution crystal structure of the *Drosophila melanogaster* alcohol-binding protein LUSH (26), and could such motifs be used to find other alcohol targets in brain? Despite these exciting findings, it seems highly likely that the clinically relevant behavioral effects of alcohol will involve multiple CNS targets; however, teasing these apart may be more feasible than once thought.

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