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$GABA_A$ Receptor Subtypes: the 'One Glass of Wine' Receptors

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Abstract

This review discusses evidence for and apparent controversy about, $GABA_A$ receptor subtypes that mediate alcohol effects experienced during social drinking. $GABA_A$ receptors that contain the β3 and δ subunits were shown to be enhanced by alcohol concentrations that mirror the concentration-dependence of alcohol responses in humans. A mutation (α6R100Q) previously found in alcohol non-tolerant (ANT) rats in the cerebellar $GABA_A$ receptor α6 subunit is sufficient for increased alcohol-induced ataxia in rats homozygous for this mutation (α6-100QQ) and further increases alcohol-sensitivity of tonic GABA currents (mediated by α6βδ receptors) in cerebellar granule cells of α6-100QQ rats and in recombinant α6R100Qβ3δ receptors. This provided the first direct evidence that these types of receptors mediate behavioral effects of ethanol. Furthermore the behavioral alcohol antagonist Ro15-4513 specifically reverses ethanol enhancement on α4/6β3δ receptors. Unexpectedly, native and recombinant α4/6β3δ receptors bind the behavioral alcohol antagonist Ro15-4513 with high affinity and this binding is competitive with EtOH, suggesting a specific and mutually exclusive (competitive) ethanol/Ro15-4513 site which explains the puzzling activity of Ro15-4513 as a behavioral alcohol antagonist.

Our conclusion from these findings is that alcohol/Ro15-4513-sensitive $GABA_A$ receptor subtypes are important alcohol targets and that alcohol at relevant concentrations is more specific than previously thought. In this review we discuss technical difficulties in expressing recombinant δ subunit-containing receptors in oocytes and mammalian cells, that may have contributed to negative results and confusion. Not only because we have reproduced detailed positive results numerous times, and we and many others have built extensively on basic findings, but also because we explain and combine many previously puzzling results into a coherent and highly plausible paradigm on how alcohol exerts an important part of its action in the brain, we are confident about our findings and conclusions. However, many important open questions remain to be answered.

1. Pharmacologically/physiologically relevant alcohol concentrations

Alcohol, besides caffeine and nicotine, is by far the most widely used legal drug. Because of alcohol's effects to impair reaction time, judgment, motor coordination, etc., societies have put legal limits on blood alcohol concentrations at which people are allowed to drive cars (0.05 % w/v in most European Union countries and 0.08% throughout the US). These limits correspond

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to blood concentration of about 11 mM (0.05% w/v) and 17 mM (0.08 % w/v). These concentrations serve as landmarks when we talk about pharmacologically/physiologically relevant alcohol concentrations. Blood alcohol concentrations of more than twice the US legal driving limit (34 mM) could be considered exceeding the normal range, resulting in considerable loss of control and frequently vomiting (as a sign of, and protective response to, acute alcohol toxicity). Human lethal blood alcohol concentrations are reported to range from 0.22 – 0.5 % w/v (50 – 110 mM), with a usual lethal concentration of around 80 mM (Koski et al., 2002; Gable, 2004). Unlike many other drugs, alcohol as a small amphiphilic molecule readily equilibrates across membranes, being metabolized by the intracellular enzyme alcohol dehydrogenase (Thomasson et al., 1993), and crosses the blood-brain-barrier to act at brain targets. It is therefore reasonable to assume that blood alcohol concentrations are similar to alcohol concentrations at molecular targets in the brain.

2. $GABA_A$ receptors as plausible alcohol targets

$GABA_A$ receptors ($GABA_A$Rs) have long been suspected as direct alcohol targets. In fact this has been so persuasive that many pharmacology textbook authors list ethanol as affecting $GABA_A$Rs. Behavioral evidence shows that alcohol shares many pharmacological effects with prototypical $GABA_A$R agonists like barbiturates and benzodiazepines (Liljequist and Engel, 1982; Dar and Wooles, 1985; Harris, 1990). Furthermore, benzodiazepines and barbiturates show cross-tolerance with alcohol (Le *et al.*, 1986), consistent with action on similar receptor subtypes, and benzodiazepines are widely used to treat the potential life-threatening effects of abrupt alcohol withdrawal in alcoholics (Nutt *et al.*, 1989).

With the development of modern electrophysiological recording techniques like patch clamp of single neurons in culture and in neurons in their native environment in sliced sections of animal brains it was found that most synaptic $GABA_A$Rs do not respond to relevant doses of ethanol, with a few exceptions (for review see (Weiner and Valenzuela, 2006)). Under conditions of low GABA concentrations, there were reports that alcohol at relevant concentrations enhances GABA currents in a subset of cultured neurons (Aguayo, 1990; Aguayo *et al.*, 2002) and also in certain neurons in slices (Palmer and Hoffer, 1990). In addition, some laboratories showed that a biochemical $GABA_A$R assay, using $^{36}Cl^-$ flux in cell-free membrane homogenates, sometimes called synptoneurosomes, is increased by relevant low doses of ethanol (Suzdak et al., 1986a; Allan and Harris, 1987).

Molecular cloning revealed that mammalian $GABA_A$Rs are formed by 19 homologous subunits, with five subunits forming a functional receptor/channel. The 19 subunits include three unique subunits (ρ_{1-3}) that appear to form homomeric and pharmacologically unique receptors, primarily found in the retina (Zhang et al., 2001). $GABA_A$R subunits are highly conserved in mammalian species, but differ in their molecular composition and pharmacology from ionotrophic $GABA_A$Rs found in the widely used model organisms *C. elegans* (Bamber et al., 1999; Wardell et al., 2006) and *Drosophila* (Buckingham et al., 2005). $GABA_A$Rs in *Drosophila* and *C. elegans* appear in composition and pharmacology more like $GABA_A$Rs formed by ρ subunits (formerly classified as $GABA_C$ receptors). Major mammalian $GABA_A$R subunits (α1–6, β1–3, γ1–3, δ, ε, θ) form functional pentameric receptors with the usual subunit arrangement of two alpha, two beta, and one gamma or delta subunit (Baur et al., 2006). This pentameric arrangement of three different subunits would allow for enormous diversity, although it has been argued that the number of major pharmacological subtypes in mammalian brains is limited (McKernan and Whiting, 1996; Barnard et al., 1998).

Most functional studies on effects on alcohol on $GABA_A$Rs have focused on recombinant $GABA_A$Rs composed either of only alpha and beta subunits (Mihic et al., 1997), or on the most abundant synaptic receptor subtypes composed of αβγ2 subunits. One report claimed that the

γ2L splice variant would generate $GABA_A$Rs with high alcohol sensitivity (Wafford et al., 1991). Numerous studies showed that receptors containing either the short (γ2S) or and the long (γ2L) γ2 splice variant are enhanced by alcohol (Sigel et al., 1993; Wallner et al., 2003), but both only at concentrations >60 mM, within the range of deadly concentrations in humans but not at lower, mildly intoxicating levels. That said, it remains possible that modulatory factors missing in receptors expressed in recombinant systems might be required for alcohol effects at relevant concentrations on these receptors. Such modulatory factors might include possible accessory proteins (Jacob et al., 2005), factors that ensure proper subunit assembly (Wang et al., 1999), and also possible endogenous ligands like neuroactive steroids that have potent actions on, in particular, δ subunit-containing $GABA_A$R subtypes (Stell et al., 2003).

3. δ Subunit-containing $GABA_A$Rs and tonic GABA currents respond to relevant alcohol concentrations

Classical synaptic GABAergic transmission is mediated by synaptic $GABA_A$Rs composed of abundant γ subunit-containing receptors, the majority of which are located postsynaptically opposite presynaptic GABA release sites. However, the importance of a constantly active (tonic) form of GABAergic inhibition is more and more recognized as a very important form of inhibition (Mody, 2001; Farrant and Nusser, 2005). Tonic inhibition is due to highly GABA-sensitive receptors activated by ambient GABA present in the brain thought to be in the range of 100 nM to 1 μM (Tossman et al., 1986; Richerson and Wu, 2003; Santhakumar et al., 2006). While, some tonic GABA currents can be mediated by γ2 subunit-containing receptors (Caraiscos et al., 2004), more often these currents are mediated by receptors that contain the δ subunit, shown to excluded from synapses (Nusser et al., 1998) or located perisynaptically (Wei et al., 2003). While these $GABA_A$R subtypes make only about 5% – 10% of total GABA receptors in the brain, their continuous (tonic) activity more than compensates for their low abundance (Nusser and Mody, 2002) and therefore these unique receptor subtypes are thought to play a crucial role in the regulation of neuronal excitability, local circuit activity, and network properties (Cheng et al., 2006).

Probably because of their low abundance, extrasynaptic localization, and insensitivity to classical benzodiazepines, and because of the reported difficulties in expressing these receptors in recombinant systems (see below), there are few reports on expressed recombinant δ subunit-containing $GABA_A$R (Saxena and Macdonald, 1994, 1996; Adkins et al., 2001; Wohlfarth et al., 2002; Bianchi and Macdonald, 2003). We decided to initiate studies on these unique receptor subtypes in recombinant systems to learn more about their properties. Similar to previous reports (Sundstrom-Poromaa et al., 2002), we found that GABA currents in oocytes injected with rat α4β3δ and α6β3δ receptor cDNAs are highly GABA- and EtOH-sensitive. In addition, we found that β3 and δ subunit-containing $GABA_A$Rs are enhanced by EtOH with a dose-dependence that mirrors human subjective feeling of alcohol intoxication (threshold response at 3 mM, or about 6 times lower than the US legal driving limit (Wallner et al., 2003; Hanchar et al., 2004).

As expected from these recombinant data suggesting an intrinsic alcohol site on these $GABA_A$R subtypes, there is now essentially unanimous agreement (for the only exception see (Borghese et al., 2006)) that neurons that express $GABA_A$R δ subunits have tonic currents that are enhanced by low doses of ethanol, assayed in brain slices (Carta et al., 2004; Wei et al., 2004; Hanchar et al., 2005; Liang et al., 2006; Fleming et al., 2007; Glykys et al., 2007). In agreement with our findings on recombinant δ-containing receptors and reports on alcohol-enhancement of tonic currents in brain slices, Carta et al. reported increased tonic GABA currents by EtOH at concentrations as low as 10 mM. However, Carta et al. (2004) interpreted this as being caused by increased GABA release, due to their observation that 1 μM tetrodotoxin (TTX, a blocker of voltage-gated neuronal sodium channels) abolished EtOH enhancement of

tonic currents. However, such experiments are difficult to interpret because TTX reduces extracellular [GABA], at least partly due to block of GABA release, but there are likely other important non-vesicular GABA sources (Richerson and Wu, 2003; Wu et al., 2006). In fact, in the presence of constant 300 nM GABA, 1 μM TTX did not abolish EtOH enhancement of tonic currents in cerebellar granule cells in our hands (Hanchar et al., 2005), fully consistent with direct actions of alcohol on these receptor subtypes. It should be noted that extrasynaptic receptors could be regarded as presynaptic, and some of these highly alcohol-sensitive receptors could be positioned at presynaptic nerve endings where they might contribute to the regulation of GABA release (Xiao et al., 2007). Furthermore, the ethanol effects on cerebellar granule cells were increased in rats harboring a mutation in the $GABA_AR$ α6 subunit, expressed only in the granule cells, and a constituent of the highly EtOH-sensitive α6β3δ-type receptors (Hanchar et al., 2005).

4. Difficulty in expressing δ subunit-containing $GABA_A$ receptors

It is generally agreed that most classical mammalian $GABA_ARs$ are heteropentamers composed of three different subunits as described above. A pentameric arrangement contains two α subunits, two β subunits and the fifth position is likely occupied by either the γ, δ, or the ε subunit. While in recombinant expression systems receptors are readily formed by just α and β subunits alone, it is thought that only few native $GABA_ARs$ in mammalian brain are formed by receptors composed of α and β subunits alone (Bencsits et al., 1999), although there is recent evidence that $GABA_ARs$ formed by α and β subunits mediate some forms of tonic inhibition (Mortensen and Smart, 2006). It seems therefore likely that most neurons have developed mechanisms to ensure proper formation of αβX $GABA_AR$, with X either a γ1 γ2 γ3 δ or ε subunit (with γ2 by far the most abundant subunit). There are a number of reports that show that recombinant expression systems have the propensity to form receptors with just α and β subunits even if γ, δ, ε subunit mRNAs/cDNAs are included. Therefore one has to be cautious in discussing γ, δ and ε subunit-containing receptors in expression systems, because these γ, δ and ε subunits actually might not be present in the functional receptors measured. This phenomenon has been well characterized for the γ2 subunit, and is most frequently circumvented by increasing the amount of γ2 subunit-coding nucleic acid, in order to reach homogeneous γ2 subunit-containing receptor populations that show maximal responses to classical benzodiazepine agonists like diazepam (Boileau et al., 2002). Similar problems with incorporation of the ε subunit are the likely reason for previous controversy concerning lack of anesthetic enhancement of ε subunit-containing $GABA_AR$: if the ε subunit is expressed, the $GABA_AR$ are insensitive to anesthetics, but if it is absent, the resultant αβ receptors lacking ε are enhanced by anesthetics like the neurosteroid THDOC (Davies et al., 1997; Thompson et al., 2002). Our experience with expressing the δ subunit in both oocytes as well as HEK cells suggests that the incorporation of the δ subunit into functional receptors is even more problematic than with γ2, and even with excess of δ subunit cRNA/cDNA the resulting GABA currents often appear to arise from heterogeneous receptor populations formed by both αβ and αβδ subunits (Hanchar et al., unpublished). Fortunately, receptor populations arising from α4/6β3 receptors (without δ) can be readily distinguished from alcohol-sensitive α4/6β3δ receptors based on their biophysical as well as pharmacological properties. Receptors formed by α4/6β3δ subunits show only little desensitization in the continued presence of 300 nM GABA (Wallner et al., 2003), whereas receptors formed by α4/6β3 subunits (without δ) desensitize over time when activated by 300 nM GABA (Hanchar et al., unpublished). Also, currents mediated by αβ subunits (without δ γ2 or ε) show high sensitivity to Zn^{2+} and are blocked to a considerable extent by 1 μM Zn^{2+} (Draguhn et al., 1990). In contrast, fully assembled receptors with δ, γ2 or ε subunits incorporated show decreased Zn^{2+} sensitivity (Draguhn et al., 1990; Thompson et al., 2002). It is this 1 μM Zn^{2+} -insensitive receptor population in oocytes that are injected with α4 β3 and δ subunits that is enhanced by low dose

ethanol (Hanchar et al., unpublished). There is general consensus that GABA currents mediated by αβ receptors are insensitive to relevant low EtOH concentrations (Wallner et al., 2003).

While there are numerous possibilities for failures to obtain receptors with appropriate pharmacology (e.g., clones that do not express functional proteins, "stable" cell lines that might have lost subunits during cell passages, lack of essential accessory modulatory subunits or appropriate post-translational modifications), we think that one of the main reasons for failure to see low dose ethanol effects on recombinant "α4β3δ" receptors is to recognize problems with δ subunit-incorporation into recombinant receptors (even if the δ subunit cRNA/cDNA is supplied in excess). For example Borghese et al. show a figure (Fig. 1A) with 300 nM "tonic" GABA currents from oocytes injected with α4, β3 and δ rat cRNA (with clones provided to them by us) that clearly show discernible EtOH responses at 3 mM, 10 mM and 30 mM. They say these effects are not significant relative to the total current. However, the current trace shown reveals a greater than 50% decay by desensitizing receptors, consistent with a large contamination with currents from ethanol-insensitive α4β3 receptors in this particular recording. With this dilution of real α4β3δ currents, the apparent EtOH efficacy is much lower than we obtain with oocytes that express apparently homogeneous α4/6β3δ receptor populations.

Borghese et al. show that currents in oocytes injected with rat α4β3δ cRNA show less inhibition by 1 μM Zn^{2+} than oocytes expressing α4β3 receptors. In our experience there is occasionally variation in EtOH responses from cell to cell, and 1 μM Zn^{2+} blockade and EtOH enhancement are correlated, likely indicating varying degrees of δ subunit-incorporation into functional receptors. Thus one would like to see low Zn^{2+}-sensitivity demonstrated in the same cell/oocytes, to show that δ subunit-containing $GABA_A$Rs contribute significantly to the current signal.

Human "α4β3δ" receptors in Borghese et al. (2006) produced very little GABA-evoked current, indicating possible technical problems with human receptor subunit expression and original traces shown reveal that GABA currents showed EtOH enhancement only at 300 mM. In addition, no 1 μM Zn^{2+} experiments, that would have provided evidence for δ subunit-incorporation into functional human receptors in oocytes and a "stable" mammalian cell line (human α4β3δ), are reported (Borghese et al., 2006).

The same concerns with δ subunit-incorporation into functional receptors measured by electrophysiology apply to other studies that failed to detect low dose ethanol-enhancement with recombinant receptors in mammalian (COS) cells (Yamashita et al., 2006) as well as in cultured cerebellar granule cells (Casagrande et al., 2006; Yamashita et al., 2006). Both of these reports lack appropriate Zn^{2+} controls that might provide some evidence that there is indeed δ subunit-expression. The data shown by Yamashita et al., who used 10–20 times less delta subunit-coding nucleic acids compared with our studies, are consistent with our experience that we need to supply excess δ subunit cRNA/cDNA for the formation of alcohol-sensitive (1 μM Zn^{2+}-insensitive) δ subunit-containing receptors. Because receptors formed by α and β subunits alone (without δ) are enhanced by $GABA_A$R active anesthetics like THDOC and propofol (Davies et al., 1997; Thompson et al., 2002), the enhancement of currents by anesthetics is not a proof for δ subunit-incorporation into functional receptors as implied by Yamashita et al. (2006).

Because in cultured cerebellar granule cells δ subunit-expression depends on both the development stage, being basically a postnatal subunit (Brickley et al., 2001), and on culture conditions, especially parameters like depolarization and neuronal activity (Gault and Siegel, 1997; Salonen et al., 2006) one would like to see conclusive evidence that the under chosen

recording conditions EtOH-insensitive GABA currents are indeed mediated by receptors containing β3 and δ subunits.

While we think that expression problems, in particular incorporation of δ subunits into functional receptors are the most likely reason for negative results, we cannot at this point exclude that there could be additional complexity, e.g., due to accessory subunits and post-translational modifications like phosphorylation, present in some, but not all recombinant systems and cells, that might be required to reconstitute receptors from recombinant subunits that show functional properties (like high ethanol sensitivity) that resemble those of δ subunit-containing receptors that mediate alcohol-sensitive tonic currents in neurons.

4. Interactions of Alcohol and the Benzodiazepine Behavioral Alcohol Antagonist Ro15-4513

While classical benzodiazepines (like diazepam) and alcohol show a well known synergism in their actions (Van Gorder et al., 1985; Hu et al., 1987), Hoffman La Roche scientists discovered in the early 1980s that a particular benzodiazepine called Ro15-4513 acts as a behavioral alcohol antagonist (Bonetti *et al.*, 1988). This finding generated considerable excitement when subsequently verified in large numbers of studies and in many independent laboratories (Kolata, 1986; Suzdak et al., 1986b; Lister and Nutt, 1987; Syapin et al., 1987; Hellevuo and Korpi, 1988; Lister and Nutt, 1988; Ticku and Kulkarni, 1988; Dar, 1992, 1995), but see (Hellevuo and Korpi, 1988) Given the surprising effectiveness of Ro15-4513 (and the complete ineffectiveness of the close structural analog flumazenil) in reversing pharmacologically relevant EtOH effects, it may not be surprising that there were skeptics that argued it must be the weak $GABA_AR$ blocking activity (partial inverse agonism) of Ro15-4513 that must be responsible for the alcohol antagonism (Lister and Nutt, 1987; Britton et al., 1988). However, almost all other negative modulators of GABA receptors (a.k.a., inverse agonists) do not act as alcohol antagonists (for recent review see (Wallner et al., 2006a)). Two exceptions of inverse agonists that do show alcohol antagonist actions are the Ro15-4513 structural analogs, RY080 and RY024, that also have been reported to show behavioral alcohol antagonism (McKay et al., 2004; Cook et al., 2005); these compounds also bind with high affinity to the [^{3}H] Ro15-4513/alcohol site on α4β3δ receptors (Hanchar et al., 2006).

Although anti-δ antibody-immunoprecipitated δ subunit-containing receptors were initially reported to bind the imidazobenzodiazepine [^{3}H]flumazenil with high affinity (Benke et al., 1991), the discovery that γ2 subunits are required for high affinity benzodiazepine receptors (Pritchett et al., 1989) led eventually to the dogma that δ subunit-containing $GABA_ARs$ (or any other $GABA_AR$ that does not contain γ subunits) do not contain a benzodiazepine binding site and therefore cannot be sensitive to benzodiazepine site ligands. It was therefore a surprise to find that the imidazobenzodiazepine Ro15-4513 reverses alcohol effects on α4β3δ receptors with an apparent EC_{50} of around 10 nM (Wallner et al., 2006b), suggesting a high affinity Ro15-4513 binding site on δ subunit-containing receptors. Indeed, immunoprecipitation experiments (Hanchar et al., 2006) confirmed the initial observation of imidazobenzodiazepine binding to these receptors (Benke et al., 1991) by showing that δ-containing receptors in cerebellum bind [^{3}H]Ro15-4513 with high affinity (K_d = 7.5 nM). This was verified using recombinant receptor expression in HEK cells to show that recombinant α4β3δ receptors (Hanchar et al., 2006) express ethanol-sensitive [^{3}H]Ro15-4513 binding, thereby opening the door for a molecular characterization of an important alcohol binding and modulatory site.

The observation that Ro15-4513 reverses low dose EtOH actions without effects on GABA currents in functional assays is consistent with a competitive EtOH displacement on these highly alcohol-sensitive $GABA_AR$ subtypes and binding assays with increasing EtOH concentrations lead to the predicted right shift of saturation binding curves indicative of a

competitive antagonism. Because the only difference between the alcohol antagonist Ro15-4513 and the non-alcohol antagonistic flumazenil is fluorine, instead of the larger azido moiety, at the C7 position of the benzodiazepine ring structure, the Ro15-4513 azido group is likely to occupy the ETOH site on these receptors. This view is fully consistent with reports that the C7 position is situated close to a residue critical for benzodiazepine binding (α1-H101 (rat numbering: (Dunn et al., 1999; Smith and Olsen, 2000; Berezhnoy et al., 2004). Most amazingly the homologous residue in α6 is α6R100 that we find to dramatically enhance alcohol sensitivity in recombinant α6β3δ receptors *in vitro* and *in vivo* when mutated to glutamine (Q) (Hanchar et al., 2005). Therefore, the most likely explanation is that the ethanol/Ro15-4513 binding site is a unique but "homologous" binding pocket involving α4/6R/Q100 in α4/6β3δ receptors, and therefore alcohol might be considered a benzodiazepine site ligand on a unique BZ binding site on α4β3δ receptors. This "alcohol-site" meets the general criteria for defining a drug receptor, based on comparison of *in vivo* and *in vitro* activity for a series of compounds, including the behavioral alcohol antagonist Ro15-4513. This is perhaps a surprising conclusion for a small molecule like ethanol, but nevertheless, all the evidence is consistent and coherent with the conclusion that this site mediates many low to moderate dose alcohol actions.

While there may still be some concerns whether or not inverse agonist activity of Ro15-4513 might contribute to its alcohol antagonist effect, our data combined with previous work on behavioral alcohol reversal effect of Ro15-4513 very strongly implicate unique types of $GABA_A$Rs as mediating important low dose alcohol actions (Paul, 2006). In fact the pharmacological resemblance of ethanol actions on recombinant receptors with behavioral actions - all ligands that have been previously shown to inhibit Ro15-4513's alcohol antagonism (flumazenil, β-CCE, FG7142) also have < 10 nM affinity for this binding site - provides unique evidence that EtOH/Ro15-4513-ensitive $GABA_A$Rs are important mediators of low to moderate dose alcohol actions (Paul, 2006).

6. Summary, open questions and future developments and apparent controversy

Fully consistent with the conclusion of $GABA_A$R involvement in ethanol actions from behavioral pharmacological experiments during that last three decades, our results confirm that $GABA_A$Rs are indeed important mediators of alcohol actions. However, it now appears that behaviorally relevant low dose alcohol actions might be preferentially mediated by a unique form of "nonsynaptic" GABAergic inhibition. Alcohol actions on these $GABA_A$R subunits now provide an explanation why in most studies synaptic $GABA_A$Rs did not show low dose alcohol sensitivity, while there was convincing evidence that under unusual conditions which might have preferentially activated highly GABA-sensitive receptors, $GABA_A$Rs did show appropriate alcohol sensitivity (Suzdak et al., 1986b; Suzdak and Paul, 1987; Aguayo, 1990; Reynolds et al., 1992; Aguayo et al., 2002). These alcohol-sensitive $GABA_A$R subtypes also show unique neurosteroid sensitivity (Stell et al., 2003), thereby providing a possible explanation for evidence that alcohol effects are influenced, or even partly mediated, by GABA-active endogenous neurosteroids (Morrow et al., 2001; Hirani et al., 2002). Furthermore the surprising finding that Ro15-4513 is a competitive alcohol antagonist on α4β3δ receptors now also provides a plausible explanation for the previously puzzling finding that Ro15-4513 can antagonize most behavioral alcohol actions at pharmacologically relevant doses. Our observation that the α6R100Q mutation is a frequently occurring allele in laboratory rats and leads to a further increase in EtOH potency on α6β3δ receptors (*in vivo* and *in vitro*) finally provides a plausible explanation for why the α6R100Q polymorphism has been segregated during selective breeding for alcohol-supersensitive phenotypes in three independent studies (Uusi-Oukari and Korpi, 1991; Saba et al., 2001; Carr et al., 2003).

Many open questions remain. While our data provide evidence for the involvement of cerebellar α6β3δ subunits and tonic GABA currents in cerebellar granule cells in slices, it remains to be determined if receptors other than those containing δ subunits might mediate Ro15-4513-sensitive alcohol actions and there is some evidence that this might be the case. For example, while δ subunit knock-out animals show changes in alcohol phenotypes (Mihalek et al., 2001), not all low dose ethanol actions are abolished. This might suggest the existence of alcohol/Ro15-4513-sensitive $GABA_A$ receptors that do not contain δ subunits, although there might be alternative explanations like compensatory changes that arise in global knock-outs. While most synaptic receptors do not appear to respond to relevant EtOH concentrations, there is some evidence that under certain circumstances synaptic receptors can be highly sensitive to alcohol (Akk and Steinbach, 2003; Roberto et al., 2003; Liang et al., 2006; Weiner and Valenzuela, 2006).

Also, the interesting synergistic action of classical benzodiazepine agonists (like diazepam) with ethanol (Van Gorder et al., 1985; Rudolph et al., 1999; Tauber et al., 2003) remains to be explained, because none of the classical benzodiazepine agonists binds with reasonable affinity to Ro15-4513/EtOH sites on α4/6β3δ receptors (Hanchar et al., 2006). Recombinant receptor expression should allow us to identify the alcohol/binding site on α4/6β3δ receptors, and this will hopefully provide some insight into which $GABA_AR$ subtypes can have “high affinity” enthanol/Ro15-4513 sites and which $GABA_AR$ subtypes might mediate alcohol/benzodiazepine synergism.

Further open questions are the interesting connection of alcohol actions with the adenosine receptors (Dar et al., 1983; Choi et al., 2004; Dar, 2006), protein kinases (Hodge et al., 1999; Proctor et al., 2003; Wallace et al., 2007), NMDA and AMPA receptors (Lovinger et al., 1989; Woodward, 2000), BK channels (Martin et al., 2004; Cowmeadow et al., 2006), glycine receptors (Davies et al., 2003), K_V^+ channels (Espinosa et al., 2001), and the modulation of alcohol actions via G protein-coupled receptors (Wan et al., 1996; Nie et al., 2004; Besheer et al., 2006) as well as the molecular mechanism behind of the fascinating observation of behavioral alcohol antagonism by pressure (Alkana et al., 1985; Davies and Alkana, 2001). The challenge is to find answers to these and many more important questions in the future and to integrate these findings into an increasingly comprehensive picture on which the majority of us can agree.

Abbreviations

GABA	γ-aminobutyric acid
$GABA_AR$	type A GABA receptor
BZ	benzodiazepine
EtOH	ethanol

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