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Ethanol acts directly on extrasynaptic subtypes of GABA_A receptors to increase tonic inhibition

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Abstract

Based on the similarity of ethanol intoxication to the behavioral effects of drugs known to target GABA_A receptors (GABARs) it has been suspected for decades that ethanol facilitates the activity of GABA. Even so, it has been surprisingly difficult to identify molecular targets of ethanol. Research conducted over the past several years suggests that a subclass of GABARs (those containing δ subunits) responds in a relevant concentration range to ethanol. Although δ subunit-containing GABARs are not ubiquitously expressed at inhibitory synapses like their γ subunit-containing, synaptic counterparts, they are found in many neurons in extrasynaptic locations. Here they give rise to a tonic form of inhibition that can potently suppress neuronal excitability. Studies have shown that both recombinant and native δ subunit-containing GABARs: 1) are modulated by behaviorally-relevant (i.e. low millimolar) concentrations of ethanol, 2) directly bind ethanol over the same concentration range, 3) show altered function upon single amino substitutions linked to changes in behavioral responsiveness to ethanol, and 4) are a site of action of Ro15-4513, a competitive antagonist of ethanol binding and a drug which prevents many of the behavioral aspects of ethanol intoxication. Despite such comprehensive evidence, however, the field is not free from controversy. This review evaluates published data for and against a central role of δ subunit-containing GABARs in ethanol actions and suggests future directions that might help settle points of controversy.

Keywords

alpha6 subunit; alpha4 subunit; delta subunit; alcohol; cerebellum; tonic inhibition

Introduction

Determining the precise molecular target of a psychoactive drug is notoriously difficult and for few drugs has this been more challenging than for ethanol. Considering that ethanol is the most widely used drug in society and that ethanol abuse is by far the most common form of substance abuse, understanding where and how this compound acts in the brain is one of the key challenges of neuroscience.

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It was first recognized decades ago that ethanol shares a pharmacological profile with drugs known to act on GABARs, however, experimental evidence that ethanol exerts effects on specific molecules involved in GABAergic signaling has been limited and controversial. Indeed, since the first published account suggesting that a specific GABAR subunit ($\gamma 2L$) is required for ethanol sensitivity (Wafford et al., 1991), findings in this field have been difficult for other labs to replicate and there is currently no consensus regarding specific molecules at which ethanol might act in exerting its effects on behavior.

There are several reasons why progress in identifying targets has been slow and at times contentious. First, the actions of ethanol in the brain occur at unusually high concentrations. Behavioral signs of intoxication appear only when millimolar levels of ethanol are present in blood, implying a weak receptor-drug interaction. Such low affinity renders traditional biochemical approaches, so useful in defining molecular sites of action, impractical. Second, most candidate targets for ethanol are ion channels which are complex molecules. Native GABARs (whose role in ethanol effects is the subject of this review) exist as heteropentamers composed of combinations selected from 19 homologous subunits. While stoichiometry and general rules for biosynthesis of the most common isoforms of native GABARs are becoming clearer (2 α subunits, 2 β subunits, and either a γ or δ subunit are thought to be required in the pentamer), many uncertainties remain. Third, even if the precise molecular makeup of a native GABAR were established, reconstitution of native GABARs with the desired subunit composition and spatial arrangement of subunits is problematic. Standard recombinant expression methods may not assure that multiple subunits will assemble with one another in their native spatial confirmations. Of course, issues such as accessory subunits, posttranslational modifications, and regulation by second messengers complicate matters further.

This review begins by summarizing positive evidence that δ subunit-containing GABARs are key targets for low (<30 mM) concentrations of ethanol. Much of this data has been obtained from complimentary studies of recombinant and native GABARs. Published objections to these findings are then discussed and, where possible, attempts are made to reconcile the negative findings with the supportive evidence. The review concludes with several suggestions for future experiments that might help clarify outstanding issues.

Evidence that ethanol acts on δ subunit-containing GABARs

In recombinant studies, low concentrations of ethanol enhance the function of extrasynaptic GABAR subtypes

A number of studies have shown that recombinant GABARs composed of specific combinations of subunits are special in that they respond to ethanol within a dosage range corresponding to blood ethanol concentrations achieved during moderate social consumption (Hancher et al., 2005; Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). The most common isoforms of GABARs contain γ subunits, and these receptors respond to ethanol at concentrations greater than 100 mM if at all (Wallner et al., 2006b). By contrast, GABARs composed from $\alpha 1$, $\alpha 4$, or $\alpha 6$ subunits, in combination with $\beta 3$ subunits, and a δ subunit are uniquely ethanol-sensitive. GABA-activated currents mediated by recombinant receptors of this composition are enhanced by 30 to 50% by 10 mM ethanol and by more than 100% by 100 mM ethanol (Hancher et al., 2005; Wallner et al., 2003). An example of current responses and ethanol dose response curves for GABARs composed of $\alpha 6\beta 3\delta$ and $\alpha 6\beta 2\delta$ is reproduced in Figure 1. Also displayed in this figure is the effect of a single amino acid variation in $\alpha 6$ found to occur naturally in rats. This polymorphism makes GABARs containing it even more sensitive to ethanol but only when composed with $\beta 3$ and δ subunits (Hancher et al., 2005). Cellular and behavioral ramifications of the $\alpha 6$ polymorphism make a strong case that such

receptors function under physiological circumstances and will be discussed in more detail in sections 2.15 and 2.16 below.

In neurons, low concentrations of ethanol enhance tonic but not synaptic inhibition

While the most abundant GABAR subunits of the α , β and γ subfamilies are found within symmetric postsynaptic densities in EM studies, δ subunits are confined to extrasynaptic membranes (Nusser et al., 1999; Nusser et al., 1998b; Peng et al., 2002; Wei et al., 2003). Here they are believed to be a defining component of the 'extrasynaptic' GABARs responsible for tonic inhibition (Farrant and Nusser, 2005). Numerous studies of knockout mice have suggested that GABARs containing $\alpha 1/\delta$, $\alpha 4/\delta$, or $\alpha 6/\delta$ are required for tonic inhibition (Brickley et al., 2001; Glykys et al., 2007; Nusser et al., 1999; Stell et al., 2003).

This physiological role, along with the high sensitivity of $\beta 3/\delta$ subunit-containing GABARs, leads to the prediction that tonic GABA currents, rather than phasic (i.e., synaptic) GABA currents, should be enhanced by low concentrations of ethanol. Indeed, in three neuronal types known to express $\beta 3$ and δ subunits, this is the case. Cerebellar granule cells (Hanchar et al., 2005), dentate gyrus granule cells (Fleming et al., 2007; Liang et al., 2006; Wei et al., 2004), and hippocampal interneurons (Glykys et al., 2007) exhibit tonic currents that are robustly enhanced by ethanol. These specific effects on tonic GABA currents have been observed for concentrations as low as 10 mM ethanol (Hanchar et al., 2005). To contrast, no enhancement of GABAR-mediated synaptic responses is observed at ethanol concentrations below 100 mM.

In certain brain areas, ethanol can also lead to increases in GABA release (Carta et al., 2004; Hanchar et al., 2005) which might increase tonic GABA current by an indirect, presynaptic rather than a direct action on extrasynaptic GABARs on the postsynaptic cell. However, even under conditions that prevent the presynaptic increases in GABA release, there is evidence that ethanol acts directly on native, extrasynaptic GABARs containing $\alpha 6$ subunits. In the presence of tetrodotoxin which inhibits presynaptic activity and under conditions in which GABA levels are stabilized (0.3 μ M added GABA plus GABA uptake blockers), the effects of ethanol persist (see Figure 2). Moreover, ethanol effects on tonic GABA currents are significantly larger in animals homozygous for the ethanol hypersensitive polymorphism in the $\alpha 6$ subunit (Hanchar et al., 2005). Given that this gene is expressed only in the postsynaptic granule cell, such effects must be postsynaptic. Taken together such results strongly support the conclusion that ethanol directly affects extrasynaptic GABARs on cerebellar granule cells.

Ethanol binds to recombinant and native δ subunit-containing GABARs

Arguably, the most straightforward evidence that a receptor - drug interaction occurs comes from biochemical experiments demonstrating binding of the drug to the receptor. Until recently it seemed unlikely that such evidence would be gathered for ethanol because of its relatively low apparent affinity for targets. However, Hanchar and colleagues unexpectedly discovered that a benzodiazepine derivative (Ro15-4513) binds with high affinity ($K_d \sim 7$ nM) to both recombinant and native, immunopurified $\beta 3/\delta$ subunit-containing GABARs (Hanchar et al., 2006). How was this finding relevant to ethanol actions? First, it suggested that a non-classical, and therefore largely uncharacterized, benzodiazepine binding site was present on extrasynaptic GABARs. Second, although the underlying mechanisms were mysterious and somewhat controversial, Ro15-4513 had been reported to reverse many aspects of ethanol intoxication at a behavioral level (Paul, 2006; Suzdak et al., 1986). Given its high affinity binding to putative ethanol targets and the availability of Ro15-4513 in radiolabeled form, binding experiments made it possible to test whether ethanol was capable of displacing this compound when it was bound specifically to δ subunit-containing GABARs.

Remarkably, experiments showed that Ro15-4513 is displaced in a competitive and saturable manner by ethanol with a K_d of 8 mM (Hancher et al., 2006; Wallner et al., 2006a). This was found to be the case for recombinant GABARs composed with $\alpha 4$, $\beta 3$ and δ subunits, as well as immunopurified GABARs pulled down with an antibody to the δ subunit, but not for GABARs containing γ subunits (even though Ro15-4513 binds to them with roughly equal affinity). Intriguingly, flumazenil (a.k.a. Ro15-1788), the clinically important benzodiazepine site antagonist, was also found to bind to δ subunit-containing GABARs with high affinity and to displace Ro15-4513. However, flumazenil could remain bound in the presence of high ethanol concentrations. As illustrated in Figure 3, flumazenil and Ro15-4513 share a very similar chemical structure differing only in one position where a fluorine atom (flumazenil) takes the place of a larger three nitrogen atom azido group (Ro15-4513). Based on structure activity considerations, Hancher et al. speculate that ethanol occupies the position where the azido group on Ro15-4513 interacts with the receptor. Thus, ethanol is able to bind to the GABAR at the same time as flumazenil but is competitively displaced by Ro15-4513. Coupled with the strong evidence that amino acid position 100 in $\alpha 6$ is a critical residue for benzodiazepine binding (Korpi et al., 1993; Mohler et al., 2001), and the effects on ethanol modulation of the ethanol hypersensitive polymorphism at this position (Hancher et al., 2005), these results provide a specific hypothesis for where ethanol binds to this class of GABARs. They also strengthen the idea that ethanol acts directly on δ subunit-containing GABARs rather than through an unidentified, intermediate protein.

The ethanol antagonist prevents ethanol enhancement of recombinant and native GABARs

At a functional level, the binding data make several clear predictions: 1) Ro15-4513 should prevent ethanol enhancement of δ subunit-containing GABAR currents, 2) flumazenil should not interfere with this ethanol modulation, and 3) although inactive on its own, flumazenil should relieve the ability of Ro15-4513 to act as an ethanol antagonist. Figure 3 shows that all three predictions are found to be true when tested on $\alpha 4/\beta 3/\delta$ subunit-containing GABARs expressed in oocytes (Wallner et al., 2006a). The β carboline β -CCE, another competitive, but structurally dissimilar ligand at classical benzodiazepine sites, was also found to antagonize the actions of Ro15-4513 in both binding (Hancher et al., 2006) and electrophysiological experiments (Wallner et al., 2006a). Finally, a point mutation in a transmembrane region of the $\beta 3$ subunit abolished the high dose (>100 mM) actions of ethanol, leaving a dose response relationship essentially identical to the binding curves reported in the accompanying study (Wallner et al., 2006a).

Is ethanol enhancement of native, extrasynaptic GABARs also prevented by Ro15-4513? We find that in cerebellar granule cells from $\alpha 6100^{Q/Q}$ rats the antagonist of ethanol binding does block ethanol modulation. Figure 4 summarizes previously unpublished results from 9 cells. In the presence of 300 nM added GABA, 30 mM ethanol causes a $31 \pm 7\%$ increase in tonic GABA current. Coapplication of 100 nM Ro15-4513 and ethanol to the same cells results in no change in tonic current ($1.9 \pm 9\%$ increase) ($p < 0.01$, ethanol alone vs. ethanol plus Ro15-4513). Application of 100 nM Ro15-4513 by itself to a separate group of 6 cerebellar granule cells from $\alpha 6100^{Q/Q}$ rats caused a significant increase in tonic current ($29.2 \pm 8.8\%$, $n = 6$, $p < 0.004$ as compared to no change). The finding that Ro15-4513 by itself increases tonic current argues that potentially confounding inverse agonist actions of this compound are not of concern. Thus, in line with previous work on acutely isolated and cultured neurons (Reynolds et al., 1992), we find that native $\alpha 6/\delta$ subunit-containing GABARs are also sensitive to the ethanol site antagonist.

These actions of Ro15-4513, flumazenil, and β -CCE in the binding and functional assays are striking. Not only do they provide specific information about a millimolar affinity ethanol binding site on a specific subtype of GABARs, but they shed light on the unexplained features

of Ro15-4513 activity that have been reported in numerous behavioral studies. For example, the effects of low doses of ethanol on anxiety, memory, motor behavior, and self-administration have all been reported to be reduced by this drug (discussed in (Paul, 2006). Also explained is the puzzling finding that the benzodiazepine site antagonist flumazenil prevents the anti-intoxication actions of Ro15-4513 even though by itself it is without any effect on behavior (Suzdak et al., 1986).

A single amino acid change linked to ethanol hypersensitivity in vivo renders recombinant and native GABARs more ethanol sensitive in vitro

Some of the figures and text shown to this point include data on a genetic polymorphism in the $\alpha 6$ subunit of the GABAR. This single nucleotide difference in the gene encoding $\alpha 6$ gives rise to two alleles which differ in the amino acid residue at position 100 in the $\alpha 6$ protein (position 100 is either an arginine or a glutamine). Previous work had linked one of these alleles ($\alpha 6100Q$) to behavioral ethanol hypersensitivity (Korpi et al., 1993). Korpi and colleagues found that “alcohol non-tolerant” or ANT rats, a line selectively bred to be more sensitive to the motor impairing effects of ethanol, carried the $\alpha 6100Q$ allele while their “alcohol tolerant” or AT counterparts carried the $\alpha 6100R$ allele. Subsequent studies with other independently derived lines (e.g., “Sardinian non-preferring” rats) that had been selectively bred for enhanced sensitivity to ethanol, also uncovered an accumulation of the $\alpha 6100Q$ allele (Saba et al., 2001). Interestingly, this amino acid change renders GABARs composed with $\alpha 6$ and $\gamma 2$ much more sensitive to benzodiazepines as the arginine residue in this position prevents benzodiazepine sensitivity in $\alpha 4$ and $\alpha 6$ GABARs (Korpi et al., 1993; Korpi and Seeburg, 1993; Mohler et al., 2001; Santhakumar et al., 2006). However, at the time of the original discovery of the polymorphism, the connection between this single amino acid substitution and increased behavioral sensitivity to ethanol was unclear.

Using the *Xenopus* oocyte expression system, we found that when the $\alpha 6100Q$ allele is expressed in combination with $\beta 3$ and a δ subunit, the resulting GABAR is 2-3 fold more sensitive to ethanol (Hancher et al., 2005). The increased sensitivity required that $\beta 3$ and δ be expressed since the two alleles of $\alpha 6$ behave identically in other subunit combinations (Figure 1). Thus, the inability to link $\alpha 6100Q$ to increased ethanol sensitivity in previous studies was likely due to the difficulty of expressing δ subunit-containing GABARs.

But does the polymorphism lead to alteration in the ethanol sensitivity of native GABARs? The only neurons in the nervous system that express both $\alpha 6$ and δ are cerebellar granule cells (CGCs) and tonic GABA currents in CGCs are known to rely on extrasynaptic GABARs composed of $\alpha 6$ and δ subunits (Brickley et al., 2001; Nusser et al., 1999; Nusser et al., 1995; Nusser et al., 1998b; Stell et al., 2003). Therefore, to examine the $\alpha 6$ alleles one would first need to determine whether tonic GABA currents from CGCs in brain slices were sensitive to ethanol, and then develop a strategy to test the effects of the single amino acid difference in intact neurons. With this logic we had originally intended to compare ethanol sensitivity of tonic GABA current in CGCs from ANT and AT rats. As we were developing our genotyping protocol we discovered to our surprise that the $\alpha 6100Q$ allele has a high abundance in Sprague Dawley rats. To date we have genotyped 68 rats obtained from Charles River Laboratories and find that the $\alpha 6100Q$ allele in this particular rat population has a frequency of 52%. Genotypic distributions are consistent with Mendelian inheritance. Of the 68 animals we have found 17 $\alpha 6100R/R$ rats, 31 heterozygotes, and 20 $\alpha 6100Q/Q$ rats. Indeed, the $\alpha 6100Q$ allele is found in several lines of rats (Carr et al., 2003); in fact, the codon for this allele is in the Rat Genome Sequence Consortium database (Gibbs et al., 2004). Thus, rather than being a spontaneous mutation the $\alpha 6100Q$ allele is an existing allele that has somehow been concentrated into the alcohol-hypersensitive groups in ANA, ANT, and Sardinian non-preferring lines by selective breeding (Carr et al., 2003; Korpi et al., 1993; Saba et al., 2001). In contrast to $\alpha 6100Q$, the

alcohol-hyposensitive $\alpha 6100R$ allele seems to be concentrated in the alcohol insensitive rats in all three studies. If these polymorphisms in $\alpha 6$ are unrelated to the behavioral alcohol phenotypes it is unclear why they would be segregated by the selective breeding.

The fortuitous discovery that the $\alpha 6100Q$ allele is naturally abundant makes it possible to breed heterozygous animals and compare littermate offspring homozygous for the $\alpha 6100R$ versus $\alpha 6100Q$ alleles. Of course, such a strategy cannot completely rule out the remote possibility that unknown, cosegregating genetic factors influence ethanol sensitivity, but it is clearly superior to comparisons of populations isolated from one another by more than 40 generations of selective breeding. It seems likely that such groups harbor additional genetic differences.

Electrophysiological analysis of CGCs in brain slices from the two genotypes ($\alpha 6100^{R/R}$ and $\alpha 6100^{Q/Q}$) showed that tonic GABA currents were enhanced by low millimolar concentrations of ethanol in both genotypes, and that significantly more enhancement was observed in slices from $\alpha 6100^{Q/Q}$ rats (Hanchar et al., 2005). As mentioned above, this was true even under conditions assuring that ethanol had no presynaptic effects to increase ethanol release (Figure 2). The genotypic difference in ethanol sensitivity of tonic currents indicates that at least some of the extrasynaptic GABARs in CGCs must be of the subunit composition $\alpha 6\beta 3\delta$.

In a subsequent study we also examined benzodiazepine sensitivity of synaptic currents in animals homozygous for these two alleles (Santhakumar et al., 2006). As predicted, analysis of spontaneous inhibitory postsynaptic currents (sIPSCs) in CGCs from $\alpha 6100^{R/R}$ and $\alpha 6100^{Q/Q}$ rats clearly revealed an enhanced benzodiazepine sensitivity of synaptic GABARs in $\alpha 6100^{Q/Q}$ slices. These findings confirm that some synaptic GABARs also contain $\alpha 6$ subunits. Furthermore, in analogy to our hypothesis regarding ethanol, the data offer a straightforward cellular mechanism for the enhanced behavioral sensitivity to benzodiazepines that has been thoroughly documented in ANT rats (Eriksson and Rusi, 1981; Hellevuo et al., 1989; Wong et al., 1996).

As mentioned earlier, ruling out presynaptic effects is particularly important because in the cerebellum ethanol causes a robust increase in GABA release if Golgi neurons are able to fire action potentials. Such ethanol-induced increases in GABA release can be observed as increases in the frequency of spontaneous but not miniature IPSCs recorded in CGCs (Carta et al., 2004; Hanchar et al., 2005). Intriguingly we found that the magnitude of increase in sIPSC frequency triggered by ethanol was significantly larger in the $\alpha 6100^{Q/Q}$ animals (Hanchar et al., 2005). This suggested to us that the mechanism by which ethanol increases GABA release involves $\alpha 6$ subunit-containing GABARs. In this way, the results also implicate a circuit mechanism whereby ethanol can increase GABA release by first acting on δ subunit-containing GABARs. We are actively pursuing this hypothesis.

Rats homozygous for the ethanol-hypersensitive allele in $\alpha 6$ are more severely impaired by low doses of ethanol in motor tasks

The much simpler genetic context provided by the $\alpha 6100^{R/R}$ and $\alpha 6100^{Q/Q}$ groups also made it possible to test whether differences in ethanol intoxication resulted from this amino acid substitution. Given that this polymorphism occurs in a gene expressed exclusively within a single class of cerebellar neurons we reasoned that cerebellum-dependent behaviour would be primarily affected. Furthermore, the dose dependence suggested from recombinant receptor recordings and receptor binding assays suggested that any differences would be most prominent at very low, i.e. mildly intoxicating, doses of ethanol. We found that very low doses of ethanol which leave $\alpha 6100^{R/R}$ rats unaffected significantly impaired the $\alpha 6100^{Q/Q}$ animals in the rotarod test, a classic assay for cerebellum-dependent motor coordination (Hanchar et al., 2005). Blood-alcohol concentration (BAC) assays performed on these animals showed that there was no difference in plasma concentration of ethanol between the genotypes; these

measurements also showed that the dose of 1g/kg yielded a BAC of approximately 15 mM. Notably, the genotype-dependent differences in rotarod performance disappeared at higher doses (>1.5 g/kg) of ethanol. In addition to showing that the polymorphism correlates with a higher behavioral sensitivity to ethanol, these data also argue that tonic GABA currents in cerebellar granule neurons can have a profound effect on motor behavior.

Objections to the hypothesis that δ subunit-containing GABARs contribute to low dose ethanol actions in brain

Despite the comprehensive, cross-confirmatory evidence reviewed above, four papers have reported unsuccessful attempts to extend these results (Borghese et al., 2006; Casagrande et al., 2007; Valenzuela et al., 2005; Yamashita et al., 2006). Upon simple inspection, these failures seem impossible to reconcile with the extensive evidence implicating δ subunit-containing GABARs in ethanol actions. We remain puzzled by these reports as we have repeated all of the key experiments (many are replicated by different experimenters in subsequent publications) and find the results to be identical to those in our original reports. Of course, it is important to point out that failed experiments are difficult to interpret and even more difficult to troubleshoot when they have been done in another lab. That said, below we carefully review the methods and data in the prior studies identifying differences between those that yielded positive results from those that report negative results. In some instances the differences can clearly account for why experiments failed while in others we are left to speculate.

Recombinant experiments with δ subunit-containing GABARs

Two publications (Borghese et al., 2006; Yamashita et al., 2006) report that $\beta 3/\delta$ subunit-containing GABARs are not sensitive to ethanol in the low millimolar range of concentrations. The first was a collaborative effort from several groups in which they examined transient expression of rat and human isoforms of $\alpha 4/\beta 3/\delta$ GABARs in *Xenopus* oocytes and made use of L(tk-) cells constructed with the intent that $\alpha 4$, $\beta 3$, and δ subunits be stably expressed (Borghese et al., 2006). We view their main conclusion as questionable in several respects. First, although they claim to have replicated our prior work, there are clear indications that the GABARs they studied are not the same GABARs as in the positive studies described above. Basic parameters of the expressed receptor pool - the average sizes of current, EC_{50} values, and relaxation dynamics upon GABA application - are notably different. For example, in the Borghese et al. study, the average GABA-induced current recorded 8 days after injection of $\alpha 4/\beta 3/\delta$ into oocytes was 3800 nA, nearly as large a steady state current as they are able to record from $\alpha 4/\beta 3/\gamma 2$ subunit-containing receptors. In contrast, the papers reporting positive effects of ethanol observed approximately 400 nA of current 7-14 days after injection of the $\alpha 4/\beta 3/\delta$ combination in oocytes (Hancher et al., 2005; Wallner et al., 2003; Wallner et al., 2006a), much less current than can be recorded from $\alpha 4/\beta 3/\gamma 2$ GABARs (Hancher et al., 2005; Wallner et al., 2003; Wallner et al., 2006a). EC_{50} values for GABA also differ for the $\alpha 4\beta 3$ isoforms (0.6 μ M for Borghese; 22 μ M for Wallner). Lastly, the extent of current relaxation to less than 40% of the peak current (see Figure 5) is much greater than is observed in the studies that report low dose ethanol effects (Fig. 1, this paper; Fig. 3, Wallner et al., 2003; Figs. 1 and 2, Wallner et al., 2006). How might such discrepancies explain why GABARs in the Borghese study are less sensitive? Although it cannot be established with certainty, the differences raise serious doubt about whether Borghese and colleagues actually replicated the conditions in the previous work. The strong enhancement that certain modulators exert on δ subunit-containing GABARs is clearly related to the low efficacy (i.e. maximal open probability) of these GABAR subtypes (Bianchi and Macdonald, 2003; Wallner et al., 2003). It remains possible that the extremely large currents observed in the Borghese study reflect receptors in a higher efficacy state. Alternatively, despite the claims of the paper, the subunit

composition they have expressed may not be the same as in those studies reporting ethanol sensitive GABARs.

Curiously, the rat and human isoforms of GABARs within the Borghese study are also quite different from one another with regard to some of these basic parameters. Rat and human $\alpha 4\beta 3\delta$ GABARs are expected to show identical functional properties because the sequences are approximately 95% identical in protein sequence, with almost all differences found in the poorly conserved M3-M4 intracellular loops. Nonetheless, the human channels studied by Borghese et al. give rise to forty times smaller currents (Storustovu and Ebert, 2006). The basis for this large difference is unclear but it calls into question whether the rat and human GABAR isoforms, which have been studied in the same expression system, have the same subunit composition.

Even ignoring such discrepancies, it is strange that the authors reject the hypothesis that $\beta 3/\delta$ subunit-containing GABARs are not responsive to low doses of ethanol when their own figure shows otherwise. Figure 1A in Borghese et al. (reproduced as Figure 5 here) illustrates clear increases in the GABA evoked current in response to 10 and 30 mM ethanol. Such effects apparently were not observed in every recording and are not reflected in the average, but their presence raises the possibility that a fraction of the GABARs in their study were ethanol-sensitive.

The recombinant experiments in the Yamashita et al. paper focused on GABARs composed with $\alpha 6$, $\beta 3$, and δ subunits. In our view this study has an obvious technical shortcoming: they used a 2:2:1 ratio of $\alpha/\beta/\delta$ cDNAs in the transfections. In order to ensure adequate expression of δ subunit-containing GABARs, we and others transfect/inject with a five- to ten-fold abundance of δ cDNA or cRNA (Borghese et al., 2006; Hancher et al., 2006; Hancher et al., 2005; Storustovu and Ebert, 2006; Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wallner et al., 2006a). Indeed, even for $\gamma 2$ subunit-containing GABARs, transfections must be conducted with an excess of $\gamma 2$ cRNA in order to ensure that all GABARs incorporate this subunit (Boileau et al., 2002; Minier and Sigel, 2004). Thus, the lack of ethanol-sensitivity in the recombinant experiments in the Yamashita paper could simply be due to a lack of δ subunits in the receptors.

Native, extrasynaptic GABARs are not directly responsive to ethanol

Four reports have argued that native, δ subunit-containing GABARs are not directly sensitive to ethanol (Borghese et al., 2006; Carta et al., 2004; Casagrande et al., 2007; Yamashita et al., 2006). Borghese et al. show data that ostensibly conflict with previously and subsequently published reports demonstrating a sensitivity to 30 mM ethanol of tonic GABA current in dentate gyrus granule cells (Fleming et al., 2007; Glykys et al., 2007; Suzdak, 1986 #23; Liang et al., 2006; Wei et al., 2004). Comparison of these studies shows minor methodological differences, but no indication of why Borghese failed to observe sensitivity in the native GABARs. In the studies reporting sensitivity, ethanol modulation of tonic current was observed in slices from adult mice under conditions in which tonic GABA concentrations were purposefully elevated in the slices (Glykys et al., 2007; Wei et al., 2004) and in adult rats with no added GABA (Fleming et al., 2007; Liang et al., 2006). In the negative report, slices from younger mice were used, and extracellular GABA concentrations were not manipulated (Borghese et al., 2006). The recent report by Fleming et al., (2007) finds a large effect (~100% increase) of 30 mM ethanol on tonic current in adolescent dentate gyrus granule cells.

It is apparent that basic aspects of the mIPSCs are unusual in the Borghese study. The average quantal size (mIPSC amplitude) is approximately 50% larger than those recorded in a wide range of studies under similar conditions in rats (Harney et al., 2003; Naylor et al., 2005; Nusser et al., 1998a; Otis et al., 1994; Otis and Mody, 1992a; Otis and Mody, 1992b; Overstreet et al.,

2000;Shao and Dudek, 2005) and mice (Glykys et al., 2007;Payne et al., 2006;Stell et al., 2003;Wei et al., 2004). This cannot be explained by elevated GABA concentrations leading to desensitization of synaptic GABARs because GABA concentrations were not manipulated in most of these other studies. Decay rates of mIPSCs are also considerably slower than in previous studies. While these features do not point to an obvious explanation for the different results, they do indicate significant, perhaps critical differences between the preparations.

In two studies, authors examined tonic GABA currents on cultured granule cells and found variable but weak responsiveness to ethanol (Casagrande et al., 2007;Yamashita et al., 2006), one of these studies suggested that this seemed to depend on whether recordings were made in whole-cell or perforated-patch mode(Yamashita et al., 2006). In several respects it is difficult to reconcile these results with those obtained in slices (Hanchar et al., 2005;Santhakumar et al., 2006) where it is clear that tonic current is mediated by GABARs composed with $\alpha 6$ and δ (Brickley et al., 2001;Santhakumar et al., 2006;Stell et al., 2003). Certainly it cannot be assumed that in the culture dish a mature complement of GABARs develops normally during the 7-14 day time period these cells spend *in vitro*. In fact, *in vivo*, the expression of $\alpha 6$ subunits has not reached adult levels until after postnatal day (PND) 35 (Brickley et al., 2001). Reflecting this, tonic GABA currents increase at least five-fold between PND7 and PND35 (Brickley et al., 1996;Brickley et al., 2001;Wall and Usowicz, 1997). Thus, even assuming development in the culture dish proceeds as in the brain, the tonic current has only reached one-third of its adult level in the oldest cultures studied.

Yet another problem is that culture conditions dramatically alter the expression levels of both $\alpha 6$ and δ subunits (Martikainen et al., 2004;Salonen et al., 2006). Even the ability to detect δ subunit protein on Western blots does not indicate that it is functional because in culture much of the protein is sequestered intracellularly (Follesa et al., 2005). Despite these concerns, the authors in the studies performed no direct assays for the presence of δ or $\alpha 6$ subunits (Casagrande et al., 2007;Yamashita et al., 2006). While Yamashita et al., did perform experiments with the $\alpha 6$ selective drug furosemide, this compound is not selective for δ subunit-containing GABARs (Tia et al., 1996) and so the sensitivity could simply indicate the presence of $\alpha 6/\gamma 2$ subunits.

A third paper recorded from cerebellar granule cells in acute slices and described robust increases in tonic current in response to doses of 20 mM ethanol and higher (Carta et al., 2004). However, contrary to the interpretation of Hanchar et al., they attribute this enhancement of tonic current entirely to an increase in GABA release. The key finding leading them to this interpretation is that they do not detect ethanol-induced increases in tonic current in the presence of tetrodotoxin which blocks the stimulatory effect of ethanol on the presynaptic GABA-releasing neuron.

Several of our own results conflict with this picture. First, we find that increases in tonic current can be detected in tetrodotoxin (Hanchar et al., 2005). Second, rats homozygous for the ethanol-hypersensitive allele of $\alpha 6$, in addition to exhibiting increased benzodiazepine sensitivity for synaptic receptors (Santhakumar et al., 2006), also show larger increases in tonic current in response to ethanol (Hanchar et al., 2005). Since $\alpha 6$ is expressed only in granule cells and the mutation leads to an increased ethanol sensitivity in recombinant receptors, it seems most plausible that ethanol is exerting a direct postsynaptic effect. Third, we found that the magnitude of increase in GABA release (measured by changes in sIPSC frequency) was larger in animals homozygous for the ethanol hypersensitive allele (Hanchar et al., 2005), suggesting that even the presynaptic effect may arise indirectly from ethanol action on an $\alpha 6/\delta$ subunit-containing GABAR. Finally, Ro15-4513, the competitive antagonist at the ethanol binding site, blocks the ability of ethanol to increase the tonic current (Figure 4).

Why were Carta et al. unable to see this increase in TTX? Here we can only speculate, but, once again, differences in the experimental conditions may be critical. For the experiments in tetrodotoxin we added 300 nM GABA and inhibited GABA uptake in order to increase the size of the baseline tonic current and prevent fluctuations in GABA concentration. We reasoned that these manipulations would ensure a sizable tonic current and be more likely to reveal the effects on GABARs if the receptors were intrinsically sensitive to ethanol. In part we feel this is justified because tonic GABA current in brain slices is almost certainly smaller than tonic currents *in vivo* (Chadderton et al., 2004; Santhakumar et al., 2006). In any case, this positive result, coupled with the new data presented in Figure 4, has a more straightforward interpretation than the negative one.

The ethanol hypersensitive mutation has no effect on native GABARs

In response to our report comparing $\alpha 6100^{R/R}$ and $\alpha 6100^{Q/Q}$ rats, the same group went on to test ethanol effects in two lines of rats selectively bred to be differentially sensitive to ethanol (Valenzuela et al., 2005). These lines were chosen because they accumulate the two $\alpha 6$ alleles such that ANT rats are typically homozygous for the ethanol hypersensitive allele, $\alpha 6100^Q$, while AT rats are homozygous for $\alpha 6100^R$ (Korpi et al., 1993). They report that in these two groups of rats ethanol increases tonic current in cerebellar granule cells to the same extent. They also saw no difference in the extent to which ethanol increases sIPSC frequency (i.e. GABA release).

Previously, we responded in some detail to these findings (Otis et al., 2005), and so here it is appropriate to summarize our main objection: given the likelihood that many other ethanol-related genes segregate during the 40 generations of selective breeding, this is a suspect test of whether the $\alpha 6$ polymorphism affects ethanol sensitivity of a native GABAR. By comparison our strategy allowed us simply to distinguish rats by genotype, breed heterozygotes, and test littermates homozygous for either allele. This strikes us as a more easily interpreted experiment. Indeed, this approach has shown us that the genotypes differ at a cellular level in their responsiveness to benzodiazepines (Santhakumar et al., 2006) as well as to ethanol (Hancher et al., 2005) and we conclude that these cellular mechanisms play important roles in the different behavioral responsiveness to benzodiazepines (Wong et al., 1996) and ethanol (Hancher et al., 2005). It should also be noted that, unlike the Valenzuela study, our experiments provide an explanation of why the two $\alpha 6$ alleles co-segregate so reliably during the selective breeding in several lines of rats (Carr et al., 2003; Korpi et al., 1993; Saba et al., 2001). If $\alpha 6100^Q$ has nothing to do with ethanol sensitivity it is puzzling why it accumulates in ethanol hypersensitive and is excluded from ethanol hyposensitive rats in various breeding paradigms.

Suggestions for future studies

While a review is rarely able to settle all outstanding issues, especially with regard to a complicated and controversial topic of this sort, such an effort does hold the possibility of clarifying key issues and suggesting critical future experiments. On the bright side, there is detailed, substantial, and accumulating evidence that extrasynaptic GABARs are responsive to low doses of ethanol. In addition, the negative findings reviewed here, although confusing, have come from experiments that have not replicated conditions in the original experiments. Indeed, in many cases there are specific concerns that provide straightforward explanations for why the experiments were not successful. But the important question remains: what can be done to settle the issue of whether δ subunit-containing GABARs play a key role in ethanol actions?

Here we have a few suggestions. First, care must be taken in recombinant studies to ensure the molecular composition of GABARs is as intended. Ideally this would involve a tag accessible only when the receptor is present on the surface membrane. Second, analysis of the ethanol

sensitivity of native GABARs, along with determination of their molecular makeup, should be a priority. Third, *in vivo* manipulations of key components of hypothesized ethanol-sensitive GABARs subtypes should be attempted and thoroughly studied. Special attention should be paid to mildly intoxicating concentrations of ethanol in behavioral analysis of such animals. Considering the success that knock-in genetic strategies have shown in delineating the molecular mechanisms of benzodiazepine and anesthetic sensitivity (Mohler et al., 2001), there is reason to be hopeful that similar approaches will prove fruitful in understanding ethanol action.

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Figure 1. GABARs composed of $\alpha 6$, $\beta 3$ - and δ subunits are sensitive to low millimolar ethanol concentrations

(a) GABARs of the indicated subunit compositions have been expressed in *Xenopus* oocytes and activated by 300 nM GABA ($\sim EC_{30}$). Brief applications of ethanol at the indicated concentrations (in mM) enhance the current in a dose-dependent manner and the presence of a $\beta 3$ subunit imparts higher ethanol sensitivity. Also displayed are responses from GABARs composed with the two alleles of $\alpha 6$ that naturally occur in rats, $\alpha 6$ -100R and $\alpha 6$ -100Q. (b) Dose-response curves for ethanol. Note that $\beta 3$ and δ -subunits are required for $\alpha 6$ 100Q, the 'ethanol-hypersensitive' allele, to exert an effect. This figure was originally published in (Hancher et al., 2005) and is reproduced here with permission.

Figure 2. Low millimolar ethanol concentrations enhance tonic GABA current

Tonic currents recorded from cerebellar granule cells in brain slices prepared from rats of the genotypes (a) $\alpha 6$ -100RR or (b) $\alpha 6$ -100QQ show that 10 mM ethanol directly enhances native GABAR function. These increases are not due to increased GABA release because the recordings have been made under conditions in which presynaptic action potentials and GABA uptake are blocked. (c) Summary data demonstrate significant increases in tonic current in both genotypes with larger enhancements in $\alpha 6$ -100QQ animals. Reproduced with permission from Hancher et al., 2005.

Figure 3. Ro15-4513 antagonizes ethanol effects on recombinant GABARs containing $\beta 3$ and δ subunits and this antagonism is prevented by flumazenil or β -CCE

(a) A recording from a *Xenopus* oocyte expressing $\alpha 4\beta 3\delta$ shows currents evoked by 300 nM GABA that were potentiated by 30 mM ethanol. This potentiation is blocked by 100 nM Ro15-4513. In the continuous presence of 300 nM GABA, 30 mM ethanol, and 100 nM Ro15-4513, the BZ site ligands flumazenil (300 nM), β -CCE (300 nM), flunitrazepam, and DMCM (each 1 μ M) were sequentially applied to test whether they reverse Ro15-4513's ethanol antagonist effects. At the end of the recording, 30 μ M bicuculline was used to block the GABA-induced current. (b) Chemical structures of Ro15-4513 and flumazenil show that they differ only at one single position in the molecule. Ethanol is hypothesized to occupy this position and in doing so can occupy a binding site at the same time as flumazenil. Reproduced with permission from Wallner et al., 2006a.

Figure 4. The ethanol site antagonist Ro15-4513 blocks the ethanol-induced enhancement of tonic GABA current in cerebellar granule cells

To the left are displayed 30 second duration epochs of membrane current from an example granule cell under the indicated conditions. Corresponding all-points histograms of membrane current are displayed along the vertical axis in the middle. Bar graphs to the right summarize the effects of the control condition ("G" for 300 nM GABA), 30 mM ethanol alone ("E"), and 30 mM ethanol plus 100 nM Ro15-4513 ("R"). The lower bar graph shows the average percent increase elicited by ethanol alone, and by ethanol plus Ro15-4513. Asterisks indicate a statistically significant difference ($p < 0.02$).

Figure 5. The example trace showing “ethanol insensitivity” in Borghese et al., 2006

This response, obtained from a *Xenopus* oocyte injected with cRNAs encoding rat $\alpha 4$, $\beta 3$, and δ subunits, is reproduced with permission from Figure 1A of Borghese et al., 2006. Based on the Hill equation parameters published in this paper (EC_{50} of 1.29 μ M, Hill slope of 0.84) the EC_{20} of $\alpha 4\beta 3\delta$ receptors is 247 nM. The abstract of the paper states, “In all cases, $\alpha 4\beta 3\delta$ GABARs were only sensitive to high concentrations of ethanol (100 mM in oocytes, 300 mM in the cell line).” Also of note is the difference in the extent and time course of the presteady state relaxation as compared to Figures 1 and 3.