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Studies of ethanol actions on recombinant δ -containing γ -aminobutyric acid type A (GABA_A) receptors yield contradictory results

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Abstract

The γ -aminobutyric acid type A receptors (GABA_A-Rs) display a wide variety of subunit combinations. Drugs such as benzodiazepines have shown differential effects based on GABA_A-R subunit composition. Actions of alcohols and volatile anesthetics generally do not vary markedly with subunits composition, with low concentrations of ethanol being poor modulators of these receptors. Recent studies showed $\alpha_{4/6}$ - and δ -containing GABA_A-Rs (located extrasynaptically, and responsible for tonic currents in selective brain regions) presenting high sensitivity to low concentrations of ethanol, but these results have not been obtained in other laboratories. We carried out additional experiments varying the receptor level of expression, and GABA and ethanol concentration, but no sensitivity to low concentrations of ethanol was detected. We will discuss these results and attempt an analysis of the possible causes for the discrepancies.

Keywords

Ion channel; inhibitory neurotransmission; delta subunit; allosteric modulation; *Xenopus* oocyte; electrophysiology

Introduction

Gifted with a long list of subunits (6 α , 3 β , 3 γ , δ , ϵ , π and θ , several of which possess isoforms), the pentameric γ -aminobutyric acid type A receptor (GABA_A-R) has an incredible number of potential subunit combinations, at least in theory. In practice, a comparatively reduced number of combinations have been observed, with the most abundant being $\alpha_1\beta_2\gamma_2\delta$ (Sieghart and Sperk, 2002; Whiting et al., 1999). Two combinations that have attracted attention lately are $\alpha_4\beta_{2/3}\delta$ and $\alpha_6\beta_{2/3}\delta$: even though they are not very abundant, they present a more restricted expression, both with respect to regional and neuronal localization, and a very distinctive pharmacology. Numerous studies have provided evidence supporting the coexpression of α_4 and δ subunits in brain, mainly in hippocampal dentate granule cells, thalamocortical relay neurons and outer layers of the cerebral cortex (Korpi et al., 2002; Mihalek et al., 1999; Nusser et al., 1999; Peng et al., 2002; Pirker et al., 2000; Sur et al., 1999). In cerebellum, δ is coexpressed with α_6 subunits in the granule cells (Jechlinger et al., 1998; Jones et al., 1997; Pirker et al., 2000; Tretter et al., 2001). The localization of δ -containing GABA_A-Rs is non-synaptic (Nusser

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et al., 1998; Sun et al., 2004; Wei et al., 2003). Their higher GABA affinity makes them exquisitely sensitive to the low GABA concentrations in the perisynaptic and extrasynaptic space, which produces a small tonic current, in contrast with the phasic current observed in the synapse, where GABA_A-Rs contain γ subunits (Farrant and Nusser, 2005).

Expression in heterologous systems

While studies of brain slices or homogenates can render valuable information, the characterization of the properties of a particular subunit combination is usually better achieved by expressing the subunits of interest in heterologous systems, therefore controlling the subunit composition of the receptor. Attractive as the approach is, it is not without caveats: the host cell may influence the receptor properties, and it may be difficult to achieve a level of expression that allows the study of the receptor of interest, among other problems. The difficulty in expressing $\alpha_{4/6}\beta_{2/3}\delta$ in oocytes (widely used for the study of other subunit combinations) probably delayed their full characterization. In our experience, expression of α_4 - and δ -containing receptors in *Xenopus* oocytes was only possible when three conditions were met: subcloning of the coding regions in a modified, high expression vector, injection of low amounts of cRNA, and recording a week or more after injection, as described in Wallner et al. (2003). Both α_4 - and α_6 -containing GABA_A-Rs were more sensitive to GABA than α_1 -containing GABA_A-Rs, and insensitive to most ligands to the benzodiazepine site (Wafford et al., 1996; Whittemore et al., 1996). For instance, diazepam potentiation was not observed in $\alpha_{4/6}$ -containing GABA_A-Rs, but it was pronounced in α_1 -containing GABA_A-Rs; flumazenil did not affect α_1 -containing GABA_A-Rs, but it increased GABA currents through $\alpha_{4/6}$ -containing GABA_A-Rs; Ro15-4513 had no effect or was a partial inverse agonist at α_1 -containing GABA_A-Rs, but was a positive modulator of $\alpha_{4/6}$ -containing GABA_A-Rs (Wafford et al., 1996; Whittemore et al., 1996). GABA_A-Rs composed of $\alpha_6\beta_3\gamma_{2L}$ or $\alpha_6\beta_3\delta$ (Saxena and Macdonald, 1996), and $\alpha_1\beta_3$, $\alpha_1\beta_3\gamma_{2L}$ or $\alpha_1\beta_3\delta$ (Fisher and Macdonald, 1997; Haas and Macdonald, 1999) were transiently expressed and their GABA responses characterized in mouse L929 fibroblasts. Later, human $\alpha_4\beta_3\delta$ subunits were stably expressed in a L(tk⁻) cell line and pharmacologically characterized, compared with $\alpha_4\beta_3\gamma_2$ (Brown et al., 2002). The $\alpha_4\beta_3\delta$ were more sensitive to GABA than $\alpha_4\beta_3\gamma_2$ GABA_A-Rs, but GABA acted as a partial agonist at $\alpha_4\beta_3\delta$, and gaboxadol (4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridin-3-ol, or THIP) showed more efficacy than GABA at this subunit combination. Desensitization following a short application of a maximally effective concentration of GABA was faster in $\alpha_4\beta_3\gamma_2$ than in $\alpha_4\beta_3\delta$ GABA_A-Rs. The sensitivity of these GABA_A-Rs to zinc was very similar, but lanthanum inhibition was more pronounced in $\alpha_4\beta_3\gamma_2$ than in $\alpha_4\beta_3\delta$. As for GABA modulators, flunitrazepam was inactive in both combinations, but $\alpha_4\beta_3\delta$ showed Ro15-4513 and bretazenil potentiation, and DMCM inhibition, while $\alpha_4\beta_3\gamma_2$ was unaltered by these drugs. Several neuroactive steroids (5 α -pregnane-3 α -ol-20-one, alphaxalone, and 5 α -pregnane-3 α ,21-diol-20-one or THDOC) were more potent modulators of GABA in $\alpha_4\beta_3\delta$ than in $\alpha_4\beta_3\gamma_2$ GABA_A-Rs. More recent studies of human and rat $\alpha_4\beta_3\delta$ expressed in oocytes replicated many of these findings (Borghese et al., 2006; Stórustovu and Ebert, 2006).

Behavioral experiments with δ -knockout mice

The generation and subsequent testing of δ -knockout mice (Mihalek et al., 1999) revealed reduced effects of neuroactive steroids; specifically, decreased sedative, anxiolytic and pro-absence seizure effects. The precise molecular basis for these differences is not clear yet. For instance, THDOC modulation of evoked and spontaneous IPSCs in thalamocortical relay neurons was not modified in δ -knockout mice (Porcello et al., 2003), but both THDOC (Vicini et al., 2002) and alphaxalone (Spigelman et al., 2003) failed to prolong spontaneous IPSCs in cerebellar and dentate granule cells, respectively, from δ -knockout mice. When the effect of THDOC was studied in dentate and cerebellar granule cells, the neuroactive steroid was much

more potent in tonic currents mediated by δ -containing GABA_A-Rs than in phasic currents (Stell et al., 2003). The tonic conductance in both dentate and cerebellar granule neurons was greatly decreased in δ -knockout mice, and its modulation by THDOC eliminated (Stell et al., 2003).

As has been observed in other GABA_A-R subunit knockout mice, the elimination of the δ subunit led to changes in the composition, and sometimes number, of the GABA_A-Rs. In the δ -knockout mice, the missing δ subunit was replaced by γ in both cerebellum granule cells (Tretter et al., 2001) and in forebrain regions (Korpi et al., 2002; Peng et al., 2002), where δ normally coexpresses with α_6 and α_4 subunits, respectively.

Compared with wild type mice, δ -knockout mice presented altered ethanol responses in several tests (reduced ethanol consumption, attenuated withdrawal from chronic ethanol exposure and reduced anticonvulsant effects of ethanol), while other tests were normal (anxiolytic response, and development of acute and chronic tolerance) (Mihalek et al., 2001). Another behavior that was not modified in δ -knockout mice was ethanol-induced sleep time, and Ro15-4513 reduced sleep in a similar way in both wild-type and δ -knockout mice (Mihalek et al., 2001). The δ -knockout mice were also characterized in an ethanol-discrimination procedure, and the authors concluded that “the delta subunit is not necessary in the mediation of ethanol-like effects of any of the GABA_A ligands tested, including sensitivity to ethanol, barbiturate, benzodiazepine, and neurosteroid discriminative stimulus effects” (Shannon et al., 2004). Delta-containing GABA_A-Rs are particularly sensitive to gaboxadol (see Section 2). Consistent with this finding, gaboxadol-induced sleep time was decreased in δ -knockout mice (Boehm et al., 2006).

Ethanol on $\alpha_{4/6}$ - and δ -containing GABA_A-Rs

The results obtained in brain slice preparations is covered in this volume by Valenzuela and others, and will not be reviewed in this article.

Sensitivity to low concentrations of ethanol

Two labs reported that δ -containing receptors expressed in oocytes were sensitive to low concentrations of ethanol, but the results diverged in several points. Sundstrom-Poromaa et al. (2002) showed that GABA-induced responses in human $\alpha_4\beta_2\delta$ were potentiated by 1-3 mM ethanol (50%), with 10 mM ethanol presenting a decreased potentiation (25%). Other combinations tested ($\alpha_1\beta_2\delta$, $\alpha_1\beta_2\gamma_{2S}$, $\alpha_4\beta_2\gamma_{2S}$) were not sensitive to these low ethanol concentrations, and other relevant combinations ($\alpha_4\beta_2$, $\alpha_4\beta_3\delta$, $\alpha_4\beta_3$, $\alpha_6\beta_3\delta$, $\alpha_6\beta_3$) were either not tested or did not express (Sundstrom-Poromaa et al., 2002).

Wallner et al. (2003) published results obtained with GABA_A-Rs constituted by rat $\alpha_{4/6}$ and $\beta_{2/3}$, with or without δ or γ_{2S} . The α and β subunits did not affect the GABA affinity, but including δ increased GABA affinity ($EC_{50} \alpha\beta \sim \alpha\beta\gamma > \alpha\beta\delta$). Positive modulators (THDOC in $\alpha_4\beta_2\delta$ and etomidate in $\alpha_6\beta_3\delta$) were capable of potentiating even maximally effective GABA concentrations, suggesting that GABA is a partial agonist at these combinations. Low concentrations of ethanol potentiated EC_{20} GABA responses when the combination included either α_4 or α_6 , β_3 and δ subunits, and 30 mM ethanol produced 75% potentiation in these combinations, but only 21% in $\alpha_{4/6}\beta_2\delta$ and none in $\alpha_{4/6}\beta_{2/3}\gamma_{2S}$. This differential sensitivity was maintained at 100 mM ethanol, with all these combinations being potentiated at similar levels by 300 mM ethanol. The combinations including only α and β subunits ($\alpha_{4/6}\beta_{2/3}$) did not show any potentiation by ethanol at any concentration.

Recent papers show that ethanol acted as a competitive inhibitor of Ro15-4513 binding on $\alpha_{4/6}\beta_3\delta$ (Hanchar et al., 2006), and that Ro15-4513 blocked ethanol potentiation of GABA-mediated currents in $\alpha_4\beta_3\delta$ GABA_A-Rs at ethanol concentrations up to 30 mM (Wallner et al.,

2006). In this last paper, flunitrazepam lacked any effect on Ro15-4513 inhibition of ethanol potentiation of GABA-mediated responses, while flumazenil reversed Ro15-4513 inhibition; neither flunitrazepam nor flumazenil blocked ethanol potentiation on its own. The authors concluded that Ro15-4513 is acting on a site different from the classical benzodiazepine binding site, where only certain benzodiazepine-like ligands can bind, but not all. Higher concentrations of ethanol seem to act on transmembrane sites of the GABA_A-R subunits, as Ro15-4513 was unable to block the ethanol potentiation, and this high alcohol potentiation was eliminated by a mutation in β_3 transmembrane domain (Wallner et al., 2006).

Lack of sensitivity to low concentrations of ethanol

Four labs tested ethanol sensitivity of α_4 - and δ -containing GABA_A-Rs in different systems, and did not observe any sensitivity to low concentrations of ethanol (Borghese et al., 2006). Our laboratory's setting most closely resembled that used by Olsen's group, since we used the same rat cDNAs, and followed their protocols. However, we did not observe in $\alpha_4\beta_3\delta$ GABA_A-Rs the sensitivity to low concentrations of ethanol. We took special care in verifying the expression of δ subunit along with α_4 and β_3 : $\alpha_4\beta_3\delta$ GABA_A-Rs presented larger GABA-induced maximal currents, and lower sensitivity to zinc. GABA affinity was not a defining characteristic: EC₅₀ GABA was similar for $\alpha_4\beta_3$ and $\alpha_4\beta_3\delta$, while $\alpha_4\beta_3\gamma_2S$ GABA_A-Rs presented a lower affinity for GABA.

The main discrepancy with previous results was in the ethanol modulation of EC₂₀ GABA responses: 30 mM ethanol produced a reliable but small potentiation (~10%) in all subunit combinations ($\alpha_4\beta_3$, $\alpha_4\beta_3\delta$, and $\alpha_4\beta_3\gamma_2S$), 100 mM induced a more pronounced, but not differential, potentiation (25-50%), and 300 mM presented a larger and distinctive pattern of potentiation: $\alpha_4\beta_3 > \alpha_4\beta_3\delta > \alpha_4\beta_3\gamma_2S$. Figures 1 and 2A through C show representative tracings, while pooled results of short applications appear in figure 2D. Interestingly, isoflurane modulation of GABA responses was similar in $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_2S$.

Experiments expressing human cRNAs in oocytes yielded similar results with respect to ethanol modulation of $\alpha_4\beta_3\delta$ GABA_A-Rs; the human subunits presented a pharmacological profile consistent with the literature, explored in more detail in a companion paper (Stórustovu and Ebert, 2006). The L(tk⁻) cell line stably expressing human $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_2S$ showed lower overall sensitivity to ethanol potentiation, but again no differences between $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_2S$ GABA_A-Rs.

Our paper also included experiments conducted in mouse hippocampal slices. Dentate granule neurons showed a tonic current blocked by bicuculline, which was not affected by 30 mM ethanol.

A more recent paper studied cerebellar granule cells and Chinese hamster ovary (CHO) cells recombinantly expressing GABA_A-Rs (Yamashita et al., 2006). In cerebellar granule cells, GABA-induced currents mediated by α_6 - and δ -containing GABA_A-Rs were decreased by 30% in the presence of 30 mM ethanol. Similarly, in $\alpha_4\beta_2\delta$, $\alpha_6\beta_2\delta$ and $\alpha_6\beta_3\delta$ GABA_A-Rs expressed in CHO cells, 30 mM ethanol had either no effect or inhibitory actions on GABA-mediated responses, while 100 mM ethanol was clearly inhibitory. Several other drugs were tested in $\alpha_6\beta_2\delta$ GABA_A-Rs expressed in CHO cells that agreed with the pharmacological profile of this combination in other systems. Another study of ethanol effect on GABA-induced currents in cerebellar granule cells in culture (Casagrande et al., 2007) found that low ethanol concentrations did not affect the slow (tail) component of GABA currents.

Further experiments in our laboratory

The methodology used was the same as described in Borghese et al. (2006). Essentially, *Xenopus laevis* oocytes were isolated and injected with cRNAs encoding the GABA_A subunits α_4 , β_3 , δ and/or γ_{2S} in the ratios 1:1:3 for α_4 : β_3 : γ_{2S} and 1:1:10 for α_4 : β_3 : δ , unless otherwise indicated (the injected amounts of α_4 and β_3 were always 0.4 ng each per oocyte). Six to nine days after injection, recordings were carried out using the two-electrode voltage clamp technique.

Delta Subunit Expression Level

Several papers were published concerning the GABA_A-R ϵ subunit: one describing receptors $\alpha 1\beta 3\epsilon$ and $\alpha 2\beta 1\epsilon$ expressed in HEK293 cells as insensitive to pentobarbital (Davies et al., 1997), and two others describing receptors $\alpha 1\beta 1\epsilon$ expressed in *Xenopus* oocytes showing modulation by pentobarbital, as well as direct effects by this barbiturate (Thompson et al., 1998; Whiting et al., 1997). This controversy would be eventually resolved (Thompson et al., 2002): using $\alpha 1\beta 1\epsilon$ expressed in *Xenopus* oocytes, it was shown that the differences between the ϵ subunits were not due to amino acid sequences nor to untranslated regions, but to levels of expression determined by the vector in which the DNA encoding the ϵ subunit was inserted. Overexpression of the subunit eliminated sensitivity to pentobarbital.

This led us to pose the following question: was the δ subunit being overexpressed (leading maybe to an unusual stoichiometry in the receptor), and did this affect the ethanol modulation?

To answer this, we injected different amounts of cRNA encoding the δ subunit, keeping the amounts of α_4 and β_3 constant (0.4 ng each per oocyte). Our hypothesis was that the level of expression of δ subunit would decrease if the amount of cRNA encoding δ is reduced, and that would allow us to test ethanol sensitivity, and determine if there was any difference.

In our previous paper (Borghese et al., 2006), GABA affinity for $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3$ GABA_A-Rs was very similar (1.29 versus 0.62 μ M GABA, respectively), and we did not see any differences in the receptors that contained a lower ratio of δ subunit (Fig. 3A). However, we had previously observed a decreased GABA-induced maximal current in $\alpha_4\beta_3$ compared with $\alpha_4\beta_3\delta$ GABA_A-Rs (12-fold), and we obtained a significant decrease in maximal currents (3.7-fold) when the ratio of cRNA encoding for α : β : δ was reduced to 1:1:0.01 (Fig. 3B). Further proof of a reduced level of δ expression was the zinc sensitivity. GABA_A-Rs lacking a δ or γ subunit are very sensitive to zinc inhibition, and we had observed 90% inhibition in $\alpha_4\beta_3$ GABA_A-Rs with 1 μ M zinc, while $\alpha_4\beta_3\delta$ GABA_A-Rs were inhibited by about 20% (Borghese et al., 2006). When the δ -encoding cRNA ratio was reduced to 1:1:0.01, zinc inhibition was enhanced (Fig. 4A). However, the ethanol sensitivity remained the same, independently of the expression level of δ subunit (Fig. 4B).

Reduced GABA concentration

For many subunit combinations, as GABA concentration increases, ethanol potentiation decreases (Mihic et al., 1994). The difficulty of obtaining large currents in heterologous systems expressing $\alpha_4\beta_3\delta$ GABA_A-Rs has probably resulted in most of the literature reporting ethanol effects on currents elicited by EC₂₀ GABA. We decided to check if the inverse relationship between GABA concentration and ethanol potentiation would hold true, and if sensitivity to low concentrations of ethanol would increase if GABA concentration were diminished. We observed no significant changes in ethanol potentiation when EC₅ GABA was used (Figure 5), compared with EC₂₀ GABA (Figure 4), suggesting that there is no inverse relationship for these combinations, at least at the lower range of GABA concentrations.

Ethanol-Zinc Interaction

Zinc is accumulated by specific neurons in the CNS into synaptic vesicles, and modulates both excitatory (glutamatergic) and inhibitory (GABAergic and glycinergic) transmission (Hirzel et al., 2006; Smart et al., 2004). Discrete binding sites in $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2S}$ GABA_A-Rs have been described, and the combination $\alpha\beta$ is more sensitive to zinc inhibition than $\alpha\beta\gamma/\delta$ (Borghese et al., 2006; Hosie et al., 2003; Stórustovu and Ebert, 2006). Given its role as endogenous modulator, and the possibility of contamination of buffers through standard laboratory procedures (some latex and nitrile gloves, as well as plastic pipettes contain zinc; Rachel Phelan and John Mihic, personal communication), we decided to study if the presence of zinc could modify ethanol actions on $\alpha_4\beta_3\delta$ GABA_A-Rs. An inverse relationship between neurotransmitter concentration and zinc effect has been described, therefore we used an EC₅ GABA for our experiments.

Low concentrations of zinc (10 and 100 nM) had minimal effects on both $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_{2S}$ GABA_A-Rs, while 1 μ M zinc produced a definite inhibition on the GABA response (Figure 6A). These same concentrations of zinc were tested in the same oocytes in the presence of 30 mM ethanol, which by itself produced a small but reliable potentiation of the GABA response (Figure 6B). The presence of zinc did not modify the ethanol effect (Figure 6B): for $\alpha_4\beta_3\gamma_{2S}$ GABA_A-Rs, both the ethanol and zinc effects were significant, but the interaction was not ($F_{3,18} = 0.53$, $p = 0.67$), and the same was true for $\alpha_4\beta_3\delta$ GABA_A-Rs (interaction $F_{3,24} = 1.67$, $p = 0.20$). Therefore, the presence of zinc did not affect the ethanol modulation of GABA responses.

Possible basis for the discrepancy

We tried several experimental approaches to identify the factors that control sensitivity to low concentrations of ethanol in $\alpha_4\beta_3\delta$ GABA_A-Rs, but none of them produced a different result. So far, there is no ready and easy explanation for the discrepancy in results between eight research groups, a situation not entirely new in the ethanol field.

Differences in system used [transient expression in oocytes and CHO cells, stable cell line L (tk⁻)], clones (rat versus human) and genetic backgrounds in the studies conducted with brain slices could possibly account for the lack of reproducibility, but in one case the clones and the system used were the same, and the protocol followed closely the one employed in the first place. We will concentrate on this particular case.

Besides the lack of sensitivity to low concentrations of ethanol, other discrepancies that were apparent were differences in a) maximal GABA responses, b) affinities for subunit combinations, and c) desensitization rate. Point a: in Wallner et al. (2006), the authors made a reference to our maximal GABA responses being five times higher than the currents they obtained. We were not able to do that comparison, because the authors did not publish their maximal currents. However, the EC₂₀ GABA tracings shown in their papers (Wallner et al., 2003; Wallner et al., 2006) were approximately of the same magnitude of ours (Figure 1 and 2C), and the EC₂₀ GABA responses in our experiments involving ethanol modulation ranged from 300 to 1800 nA (mean \pm SEM = 1060 ± 100 nA), and there was no correlation between the magnitude of the GABA response and the ethanol effect. Therefore, the differences observed in ethanol sensitivity were not related to the magnitude of the current itself, and we are not sure if the maximal currents could be so divergent. As for the GABA-mediated currents in oocytes injected with human cRNAs, they were considerable smaller than with the rat counterparts (Borghese et al., 2006), but the human $\alpha_4\beta_3$ and $\alpha_4\beta_3\delta$ expressed in oocytes have been pharmacologically characterized, and all the subunits were present (Stórustovu and Ebert, 2006). Point b: in our paper, the relative order for EC₅₀ GABA was $\alpha_4\beta_3 \sim \alpha_4\beta_3\delta < \alpha_4\beta_3\gamma_{2S}$, which is the order observed for these and similar combinations (Brown et al., 2002; Fisher and

Macdonald, 1997; Stórustovu and Ebert, 2006; Thompson et al., 1997). Therefore, even though there was a shift to the right for $\alpha_4\beta_3\delta$ compared with Wallner et al. (2003), we feel that the overall results were consistent with the literature. Point c: the desensitization rate for $\alpha_4\beta_3\delta$ was higher in our preparation than in Wallner et al. (2003,2006), but $\alpha_4\beta_3\delta$ always presented less desensitization than $\alpha_4\beta_3\gamma_{2L}$ (Figure 1).

The δ -knockout mice could provide interesting insight into the situation. If ethanol actions at low concentrations are mediated by $\alpha_{4/6}\beta_3\delta$ GABA_A-Rs, which ethanol behaviors are affected when these receptors are absent? At 1.5 g/kg i.p. (the lowest dose used), ethanol hypothermic and anxiolytic effects were similar in wild-type and δ -knockout mice (Mihalek et al., 2001). Moreover, deletion of the δ subunit did not modify the acquisition of an ethanol/saline discrimination (Shannon et al., 2004). Ethanol consumption was decreased in δ -knockout mice, albeit the decrease was less in females (Mihalek et al., 2001). More testing is necessary, but so far, the behaviors mediated by δ -containing GABA_A-Rs seem to be limited. Even though it has been proposed that ethanol anesthetic effects are mediated by different binding sites in the GABA_A-R transmembrane domains, it is surprising that ethanol-induced sleeping time was not altered in δ -knockout mice, and was unaltered by Ro15-4513 (Mihalek et al., 2001), when gaboxadol (selective agonist for δ -containing GABA_A-Rs) showed a decreased sleeping time in δ -knockout mice (Boehm et al., 2006).

Post-translational modifications can greatly affect the behavior of GABA_A-Rs by altering channel currents and/or trafficking (Brandon et al., 2002; Luscher and Keller, 2004). It has been suggested that protein kinase A-induced phosphorylation of β_3 subunit in $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_{2L}$ could modify currents through these GABA_A-Rs, by respectively decreasing and increasing fast desensitization (Tang and Macdonald, 2005). Differences in the phosphorylation state of the GABA_A-R could in principle account for differences in the ethanol sensitivity, although it is difficult to understand how phosphorylation state could vary between laboratories for very similar preparations.

Ten laboratories have published results of low concentrations of ethanol effects on δ -containing receptors in heterologous systems, cultured neurons and slice preparations. Three have observed potentiation: Smith's and Olsen's for recombinant receptors (although for different combinations), and Mody's for slice preparations. Laboratories that have seen no effect or even inhibition include Wafford's, Ebert's, Narahashi's, Robello's and Harris' for recombinant receptors and cultured neurons, and Lambert's and Valenzuela's for slice preparations. We conclude that there are factor/s still unaccounted for that determine the sensitivity of δ -containing receptors to low concentrations of ethanol.

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Figure 1

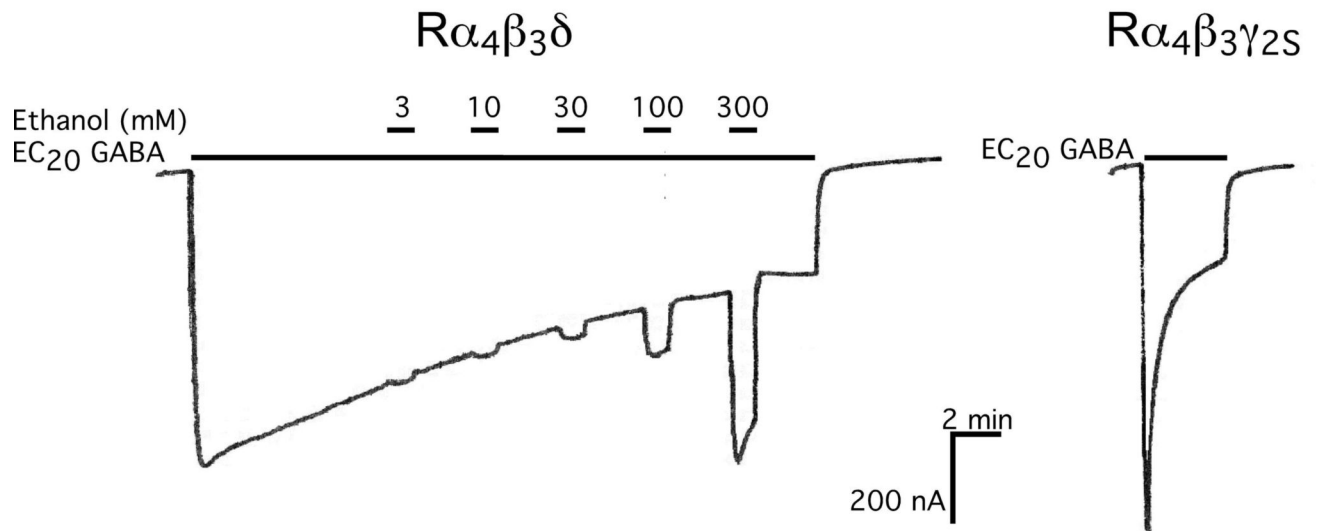


Figure 1. Tracings showing the ethanol effect on EC₂₀ GABA responses (long application) in rat $\alpha_4\beta_3\delta$ expressed in oocytes. The faster desensitization of the GABA current in rat $\alpha_4\beta_3\gamma_2\delta$ is shown for comparison. Reproduced by permission of The American Society for Pharmacology and Experimental Therapeutics, adapted from Borghese et al. (2006).

Figure 2

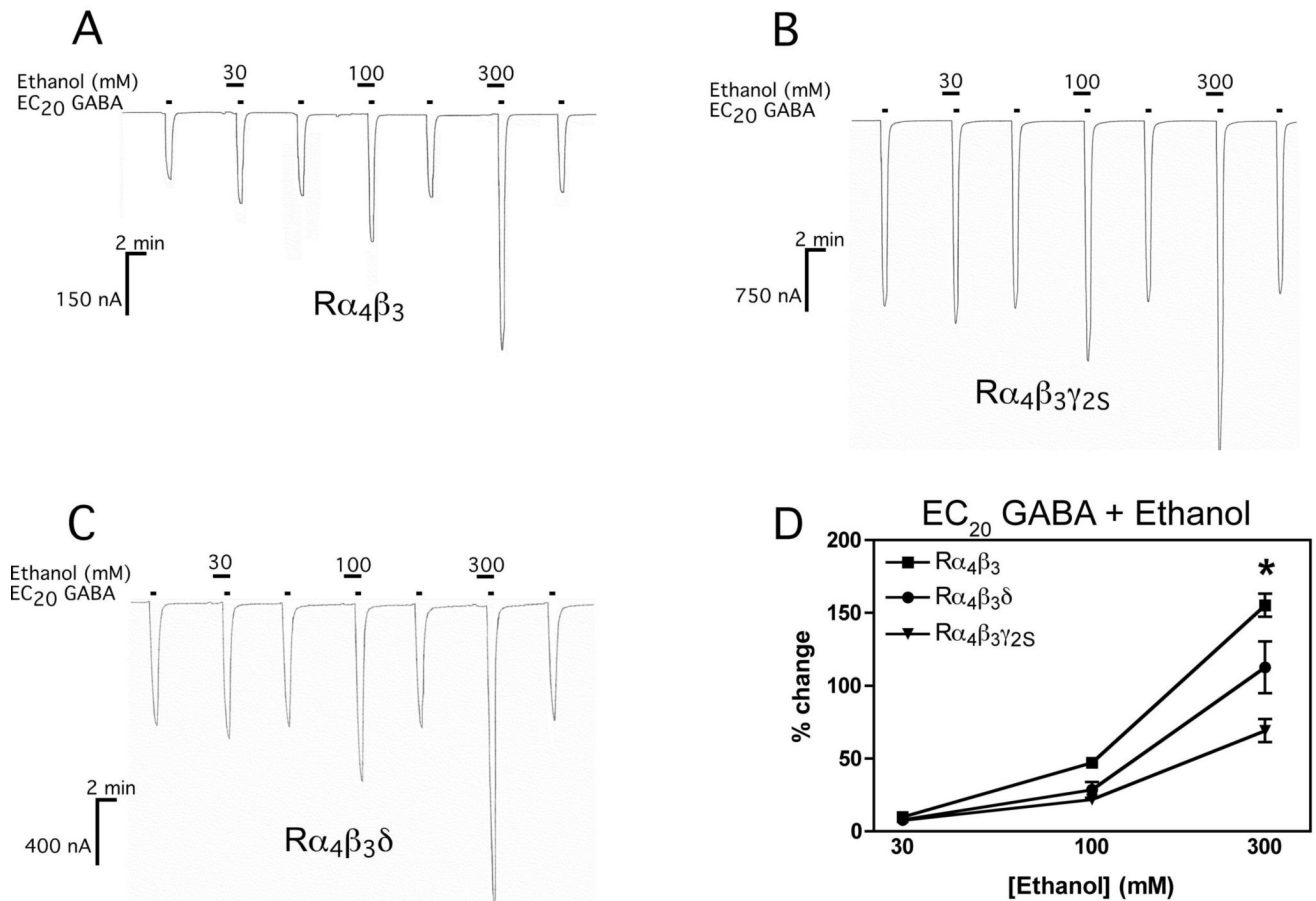


Figure 2. Tracings and graph showing the ethanol effect on EC₂₀ GABA responses (short application) in rat $\alpha_4\beta_3$ (A), $\alpha_4\beta_3\gamma_2s$ (B) and $\alpha_4\beta_3\delta$ (C) expressed in oocytes. (D) Pooled results; values are mean \pm S.E.M., n= 6-8, * p< 0.05 versus other subunit combinations at 300 mM ethanol. Reproduced by permission of The American Society for Pharmacology and Experimental Therapeutics, adapted from Borghese et al. (2006).

Figure 3

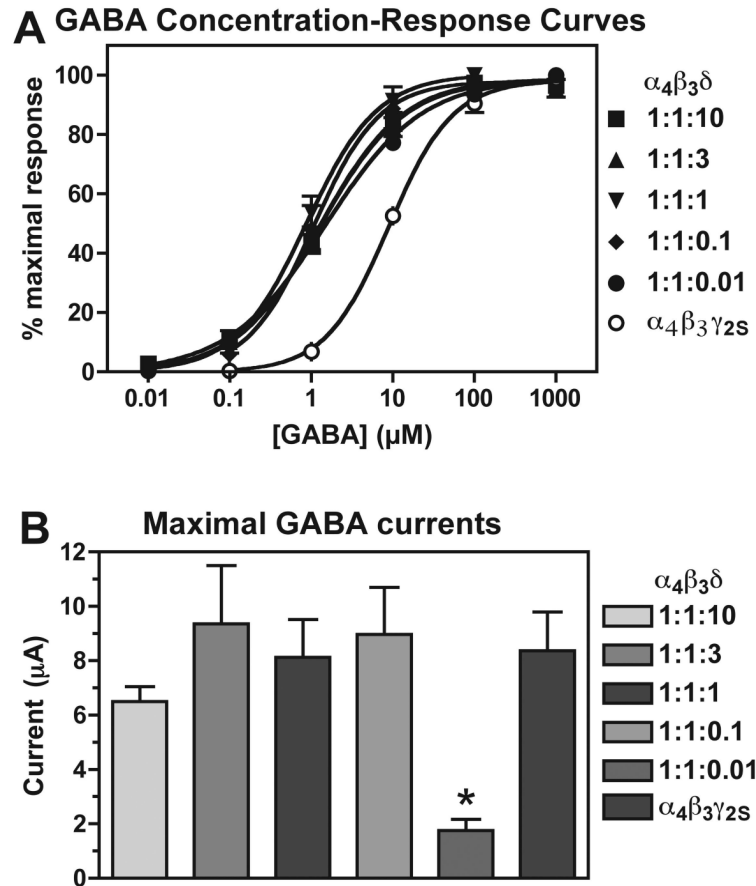


Figure 3. GABA responses of rat subunits injected in different ratios in oocytes. GABA concentration-response curves (A) and GABA maximal responses 7-9 days after injection (B) when $\alpha_4\beta_3\gamma_{2S}$ (subunit ratio 1:1:3) and $\alpha_4\beta_3\delta$ (subunit ratio ranging from 1:1:10 to 1:1:0.01) were expressed. In A, n=3-12; in B, *p<0.01 versus $\alpha_4\beta_3\delta$ 1:1:10 (Dunnett's Multiple Comparison Test, ANOVA), n=4-25.

Figure 4

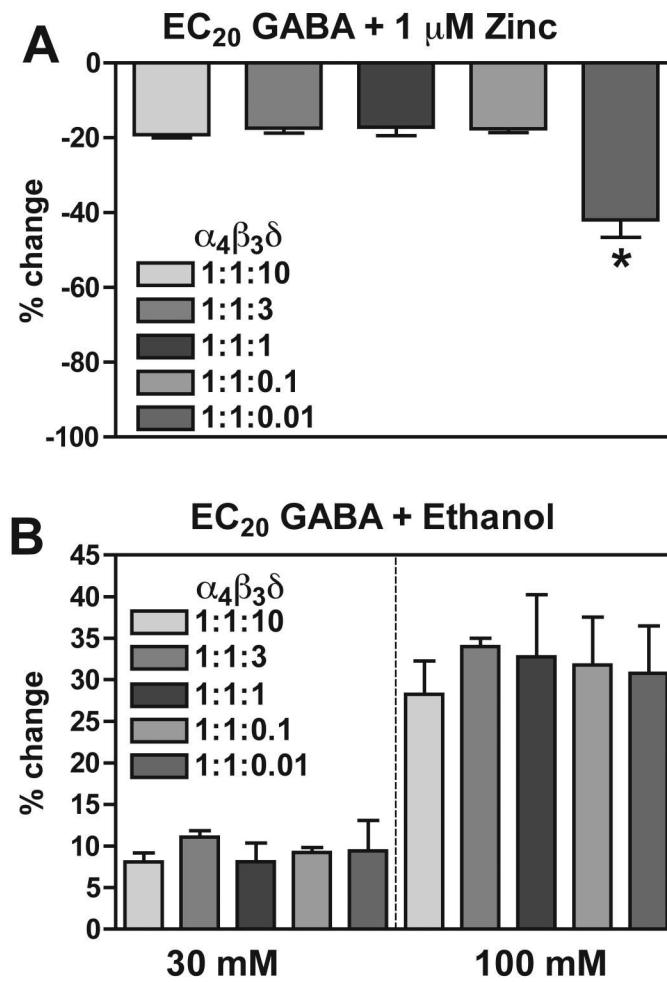


Figure 4. GABA modulation by zinc and ethanol of rat subunits injected in different ratios in oocytes. Zinc (1 μ M) inhibition (A) and ethanol (30 and 100 mM) potentiation (B) of EC_{20} GABA responses when $\alpha_4\beta_3\gamma_{2S}$ (subunit ratio 1:1:3) and $\alpha_4\beta_3\delta$ (subunit ratio ranging from 1:1:10 to 1:1:0.01) were expressed. * $p < 0.01$ (Dunnett's Multiple Comparison Test, ANOVA), $n = 3-8$.

Figure 5

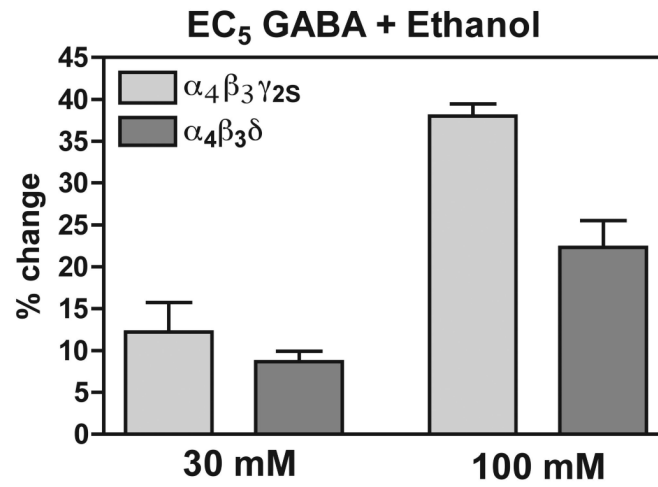


Figure 5. Modulation by ethanol of a lower concentration of GABA (EC₅) applied to rat $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_{2s}$ expressed in oocytes. n= 4.

Figure 6

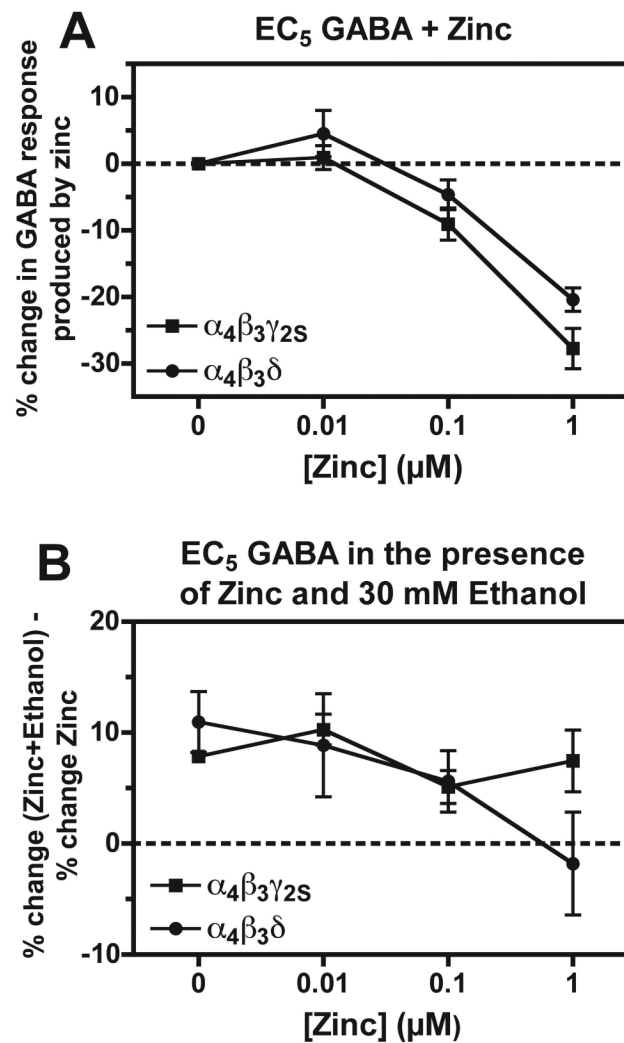


Figure 6. GABA modulation by zinc and ethanol of rat $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_2S$ expressed in oocytes. (A) Zinc (0.01-1 μ M) effect on EC₅ GABA responses. (B) Difference between the percentage of change in the EC₅ GABA response when coapplied with different concentrations of zinc (0-1 μ M) and 30 mM ethanol, and when coapplied with zinc alone at those same concentrations. N= 4-5.