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Low concentrations of ethanol do not affect radioligand binding to the δ -subunit-containing GABA_A receptors in the rat brain

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Abstract

In the present study, we investigated the co-localization pattern of the δ -subunit with other subunits of GABA_A receptors in the rat brain using immunoprecipitation and Western blotting techniques. Further, we investigated whether low concentrations of ethanol affect the δ -subunit-containing GABA_A receptor assemblies in the rat brain using radioligand binding to the rat brain membrane homogenates as well as to the immunoprecipitated receptor assemblies. Our results revealed that δ -subunit is not co-localized with γ_2 -subunit but it is associated with the α_1 -, α_4 - or α_6 -, β_2 - or/and β_3 -subunit(s) of GABA_A receptors in the rat brain. Ethanol (1–50 mM) neither affected [³H]muscimol (3 nM) binding nor diazepam-insensitive [³H]Ro 15–4513 (2 nM) binding in the rat cerebellum and cerebral cortex membranes. However, a higher concentration of ethanol (500 mM) inhibited the binding of these radioligands to the GABA_A receptors partially in the rat cerebellum and cerebral cortex. Similarly, ethanol (up to 50 mM) did not affect [³H]muscimol (15 nM) binding to the immunoprecipitated δ -subunit-containing GABA_A receptor assemblies in the rat cerebellum and hippocampus but it inhibited the binding partially at a higher concentration (500 mM). These results suggest that the native δ -subunit-containing GABA_A receptors do not play a major role in the pharmacology of clinically relevant low concentrations of ethanol.

Keywords

GABA_A receptors; δ -Subunit; Immunoprecipitation; Western blot; Co-localization; Radioligand binding

1. Introduction

GABA_A receptors are heteropentameric membrane proteins derived from various subunits such as α_{1-6} , β_{1-3} , γ_{1-3} , δ , ϵ , θ , π and ρ_{1-3} , and differ in subunit composition, function and sensitivity to various drugs (see Mehta and Ticku, 1999a;Whiting, 2003). The molecular mechanisms underlying the expression of various GABA_A receptor subunits and the assembly of subunit combinations to form functional receptors are unknown. To elucidate the cellular location, subunit composition and functional role of native GABA_A receptors is important in order to develop better drugs. Majority of the native pentameric GABA_A receptor assemblies are believed to contain two α , two β and one γ or δ subunits (Benke et al., 1991;Mertens et al., 1993;Quirk et al., 1994,1995;Araujo et al., 1998; see Mehta and Ticku, 1999a;Whiting, 2003), and the most common subunit combination for GABA_A receptors in the brain is

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$\alpha_1\beta\gamma_2$ (Fritschy et al., 1992; De Blas, 1996). The δ -subunit-containing GABA_A receptors are not found in abundance in the brain but the knockout of δ -subunit in mice reduced ethanol consumption, ethanol-withdrawal hyperexcitability as well as anticonvulsant effect of ethanol (Mihalek et al., 2001). However, there were normal anxiolytic, sedative, hypothermic and development of acute/chronic tolerance responses to ethanol in these mice (Mihalek et al., 2001). Further, the discriminatory stimulus effect of ethanol was found to be similar in the δ -subunit knockout versus wild-type mice (Shannon et al., 2004). It has been reported recently by many investigators that the δ -subunit-containing GABA_A receptors are involved in the pharmacological actions of clinically relevant low concentrations of ethanol (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004; Hancher et al., 2004; Glykys et al., 2007). Further, it has been proposed that ethanol-induced motor impairment is mediated through the extrasynaptic δ -subunit-containing GABA_A receptors in the cerebellar granules (Hancher et al., 2005). However, there are also reports indicating that the δ -subunit of GABA_A receptors does not confer sensitivity to clinically relevant low concentrations of ethanol (Borghese et al., 2006; Yamashita et al., 2006; Casagrande et al., 2007). These conflicting reports may be due to differences in the receptor subunits combinations or cell type. Thus we investigated the co-localization pattern of the δ -subunit with major subunits (α_1 , α_4 , α_6 , β_2 , β_3 and γ_2) of GABA_A receptors in the rat brain under identical conditions using immunoprecipitation and Western blotting techniques prior to using various brain regions for investigating the effect of low concentrations of ethanol on the radioligand binding to the membrane homogenates as well as to the immunoprecipitated δ -subunit-containing GABA_A receptor assemblies. Notably, previous studies utilized either behavioral models or patch-clamp technique in recombinant expression system, cultured neurons and tissue slices to explore this aspect.

2. Results

2.1. Co-localization pattern of δ -subunit with other subunits of GABA_A receptors in the rat brain

Various amounts of tissue protein (1500 μ g, 1000 μ g, 500 μ g and 100 μ g) were immunoprecipitated, dot blotted and immunostained using antibodies for the GABA_A receptor subunits (α_1 , α_4 , α_6 , β_2 , β_3 , γ_2 , and δ) as detailed in the experimental procedures. In these experiments, the antibodies for various subunits formed the immunoreaction products so as to yield their respective protein bands (Fig. 1). For subsequent experiments, 1000 μ g of the tissue protein was used as this concentration yielded the protein band of satisfactory quality. As a negative control (NC), we performed parallel experiments using non-immune IgG. No protein-band was detected in these control experiments since the non-immune IgG did not react with the polypeptides of the GABA_A receptor subunits (Fig. 1). Further, pre-incubation with the peptide (20 μ g/ml) for the δ -subunit GABA_A receptors antibody blocked the immunoprecipitation in control experiments, thereby indicating the specificity of the antibody for the δ -subunit-containing GABA_A receptors.

In order to investigate the co-localization pattern of δ -subunit with other subunits of GABA_A receptors in the rat brain, the δ -subunit of GABA_A receptors was immunoprecipitated using antiserum for this subunit, which was followed by Western blot analysis using antibodies for various subunits of GABA_A receptors in the rat cerebellum (α_1 -, α_6 -, β_2 -, β_3 -, γ_2 -, and δ -subunits) or hippocampus (α_4 - and δ -subunits) depending on the abundant localization of these subunits (Wisden et al., 1992). Antibody for the γ_2 -subunit of GABA_A receptors did not react with the immunoprecipitated polypeptide of the δ -subunit following Western blotting (Fig. 2), thereby indicating an absence of co-localization of δ -subunit with γ_2 -subunit in the rat cerebellum. In contrast, antibody for the α_1 -, α_4 -, α_6 -, β_2 -, or β_3 -subunit reacted with the immunoprecipitated polypeptides of the δ -subunit following Western blotting (Fig. 2), thereby

indicating an association of δ -subunit with α_1 -, α_4 - or α_6 -, β_2 - or/and β_3 -subunit(s) of GABA_A receptors. We confirmed these results further by immunoprecipitating various subunits of the GABA_A receptors using appropriate antibodies, and followed by Western blot analysis using antiserum for the δ -subunit of GABA_A receptors. This second set of experiments revealed similar co-localization pattern of δ -subunit with other subunits of GABA_A receptors (Fig. 2). These experiments revealed the molecular mass of various subunits of GABA_A receptors as follows: α_1 = 51 kDa, α_4 = 64 kDa, α_6 = 58 kDa, β_2 = 52 kDa, β_3 = 54 kDa, γ_2 = 43 kDa and δ = 54 kDa. Notably, there was no protein band in the negative control (NC) experiments with non-immune IgG under identical conditions (Fig. 2). Similarly, there was no protein band when pre-incubated with the peptide (20 μ g/ml) for the δ -subunit GABA_A receptors antibody in control experiments (Fig. 2).

2.2. Effect of ethanol on radioligand binding to the rat brain membrane homogenate and immunoprecipitated GABA_A receptor assemblies

Ethanol (up to 50 mM) did not elicit any effect on [³H]muscimol (3 nM) and diazepam-insensitive [³H]Ro 15–4513 (2 nM) binding in the membranes from the rat cerebellum and cerebral cortex (Table 1). However, a higher concentration of ethanol (500 mM) caused partial inhibition of [³H]muscimol (3 nM) binding (7–10%) and diazepam-insensitive [³H]Ro 15–4513 (2 nM) binding (18–22%) as shown in Table 1. Similarly, ethanol (up to 50 mM) did not elicit any effect on [³H]muscimol (15 nM) binding to the immunoprecipitated GABA_A receptor assemblies derived from δ -subunit in the rat cerebellum or hippocampus, whereas a higher concentration of ethanol (500 mM) caused partial inhibition (12%–14%) as shown in Table 2.

3. Discussion

δ -Subunit is thought to co-localize with either α_4 or α_6 subunit in the native GABA_A receptor assemblies (Jones et al., 1997; Sur et al., 1999). A small population of the GABA_A receptors in the mouse cerebellum is also reported to have both δ - and α_1 -subunits (Poltl et al., 2003). GABA_A receptors derived from the δ -subunit co-localized with α_1 -subunit are reported to form functional receptors in expression systems (Wohlfarth et al., 2002; Bianchi et al., 2002) as well as in the mouse hippocampus (Glykys et al., 2007). However, δ -subunit does not co-localize with γ -subunit in the native GABA_A receptor assemblies (Quirk et al., 1994, 1995; Araujo et al., 1998). Various conflicting reports in the literature regarding the involvement of δ -subunit-containing GABA_A receptors in the pharmacology of ethanol may be due to differences in the receptor subunits combination or cell type. Our present study revealed that the δ -subunit of GABA_A receptors does not co-localize with γ_2 -subunit but it is associated with α_1 -, α_4 - or α_6 -, β_2 - or/and β_3 -subunit(s) in the rat brain.

The δ -subunit-containing GABA_A receptors are known to have high affinity for the GABA binding site agonist muscimol but lack affinity for the classical benzodiazepines (Shivers et al., 1989; Saxena and Macdonald, 1994, 1996; Storustovu and Ebert, 2006). Further, the substitution of the γ -subunit by δ -subunit is reported to produce GABA_A receptors that display high affinity for the GABA agonist muscimol but no benzodiazepine binding sites (Saxena and MacDonald 1996). However, Ro 15–4513 is reported to have high affinity for the δ -subunit-containing GABA_A receptors (Araujo et al., 1998; Hanchar et al., 2006; Yamashita et al., 2006). In view of conflicting reports regarding the involvement of δ -subunit-containing GABA_A receptors in the pharmacology of clinically relevant low concentrations of ethanol based on behavioral models (Mihalek et al., 2001; Shannon et al., 2004; Hanchar et al., 2005) or patch-clamp technique in recombinant expression system (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Hanchar et al., 2005; Borghese et al., 2006; Yamashita et al., 2006), cultured cerebellar granule cells (Yamashita et al., 2006; Casagrande et al., 2007), cerebellar slices (Hanchar et al., 2005) and hippocampal slices (Wei et al., 2004; Borghese et al.,

2006; Glykys et al., 2007), we investigated this aspect using [^3H]muscimol and [^3H]Ro 15–4513 binding to the rat brain membrane homogenates (cerebral cortex and cerebellum) as well as [^3H]muscimol binding to the immunoprecipitated δ -subunit-containing GABA_A receptor assemblies from the rat cerebellum and hippocampus. However, we did not investigate the effect of ethanol on [^3H]Ro 15–4513 binding to the immunoprecipitated δ -subunit-containing GABA_A receptors because of difficulty in quantifying it accurately due to low binding sites, which is about 6% of the [^3H]Ro 15–4513 binding sites in cerebellum (Hanchar et al., 2006) and far less in hippocampus and cerebral cortex in contrast to [^3H]muscimol binding to the δ -subunit-containing GABA_A receptors in the rat cerebellum ($\approx 20\%$, Araujo et al., 1998; see Mehta and Ticku, 1999a). Further, we did not investigate the effect of ethanol on [^3H]muscimol or [^3H]Ro 15–4513 binding in the rat hippocampal membrane homogenate due to (a) small amount of available tissue, (b) negative data in the immunoprecipitated experiments with [^3H]muscimol/hippocampus in the present study, and (c) negative data in cerebral cortex membrane homogenate which has similar δ -subunit-containing GABA_A receptor assemblies (δ - and α_4/α_1 -subunits combination). Our data indicate that ethanol does not affect the native δ -subunit-containing GABA_A receptors at clinically relevant low concentrations in the rat cerebellum (δ - and α_6/α_1 -subunits combination), hippocampus (δ - and α_4/α_1 -subunits combination) and cerebral cortex (δ - and α_4/α_1 -subunits combination), thereby supporting the view that the native δ -subunit-containing GABA_A receptors do not play a major role in the pharmacology of clinically relevant low concentrations of ethanol (Borghese et al., 2006; Yamashita et al., 2006; Casagrande et al., 2007). However, we are unable to answer the question why some investigators (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004; Hanchar et al., 2004; Glykys et al., 2007) are able to demonstrate the effect of clinically relevant low concentrations of ethanol on the δ -subunit-containing GABA_A receptors since ethanol did not elicit any effect on the δ -subunit-containing GABA_A receptor assemblies in the rat cerebellum, cerebral cortex, or hippocampus in our present study. It is possible that differences in the unknown intracellular factors/components may be responsible for the conflicting effects of ethanol on the δ -subunit-containing GABA_A receptors in different experimental models.

4. Experimental procedures

Adult male Sprague-Dawley rats (Harlan, Indianapolis IN, U.S.A.) weighing 200–250 g were maintained at a constant room temperature (22°C) on a 12-h light/12-h dark cycle. All experiments were conducted in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the National Institutes of Health. Adequate measures were taken to minimize pain or discomfort to the animals. Food and water were available ad libitum. Different regions of the rat brain were dissected, and tissues were stored at -80°C until use.

4.1. Immunoprecipitation and immunoblotting

Tissues were homogenized in ice-cold lysis buffer (50 mM HEPES, pH 7.4, 150 mM sodium chloride, 1% Triton X 100, 10 mM sodium fluoride, 1 mg/ml bacitracin, 50 mM β -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM *N*-ethyl maleimide, 1 mM Na_3VO_4 , 10 $\mu\text{g}/\text{ml}$ leupeptin). These samples were centrifuged (2300 g, 10 min, 4°C) so as to remove the supernatant containing protein. Immunoprecipitation experiments were performed as detailed earlier (Kanakura et al., 1991; Marutha Ravindran and Ticku, 2006a, 2006b; Marutha Ravindran et al., 2007). Earlier reports from our laboratory and preliminary experiments revealed that 1:1000 dilution (α_1 -, β_2 -, β_3 -, and γ_2 -subunits), 1:100 dilution (δ - and α_6 -subunits), or 1:500 dilution (α_4 -subunit) of antiserum for the GABA_A receptor subunits yields the protein band of satisfactory quality (Marutha Ravindran and Ticku, 2006a, 2006b; Marutha Ravindran et al., 2007). Antibodies for α_4 - (catalog # OPA1-04102), β_2 - (catalog # OPA1-04107), and β_3 -subunits (catalog # OPA1-04108) of GABA_A receptors were

obtained from Affinity Bio-Reagents (Golden CO, U.S.A.). These antibodies were fusion protein of MBP with the amino acid sequence from the cytosolic loop of rat GABA_A receptors. Polyclonal antibodies specific for the α_1 (AA 1–15), α_6 (AA 1–13), γ_2 (AA 1–29) and δ (AA 1–11) subunits of GABA_A receptors were procured from Alpha Diagnostics (San Antonio TX, U.S.A.). For dot blotting analysis, various amounts of the tissue protein (1500, 1000, 500, and 100 μ g) were diluted with phosphate-buffered saline 1X (PBS) to 1 μ g/ μ l, boiled in a boiling water-bath for 5 minutes to denature the proteins followed by immunoprecipitation using 30 μ l of the diluted antiserum for the GABA_A receptor subunits (α_1 , α_4 , α_6 , β_2 , β_3 , γ_2 , or δ) and 150 μ l of Protein A agarose beads (Upstate, Charlottesville VA, U.S.A.). For the negative control, 1500 μ g of protein samples were treated with 7.5 μ l (7.5 μ g) of the affinity-purified non-immune rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz CA, U.S.A.) and 150 μ l of Protein A agarose beads as detailed earlier (Marutha Ravindran and Ticku, 2006a, 2006b; Marutha Ravindran et al., 2007). To test the specificity of antibody for the GABA_A receptors δ -subunit, the peptide for δ -subunit antibody (20 μ g/ml) was added prior to incubation with the antibody so as to check whether it blocks the detection of δ -subunit. The immunoprecipitated proteins were dot blotted to the PVDF membrane (Bio-Rad, Chicago IL, U.S.A.) as described in the literature (Araujo et al., 1998). For subsequent experiments, 1000 μ g of the tissue protein was used so as to obtain a satisfactory quality of the protein bands. To study the association of δ -subunit with the other subunits of the GABA_A receptors, 1000 μ g of the immunoprecipitated protein of the GABA_A receptors δ -subunit following electrophoresis was transferred to the membrane and was incubated with primary antibody for various subunits (α_1 , α_4 , α_6 , β_2 , β_3 , or γ_2) for Western blot analysis. Then the membrane was washed several times, and peroxidase-coupled secondary antibody (anti-mouse IgG/anti-rabbit IgG; New England Biolabs, U.S.A.) was added and incubated for 1h. The membrane was washed and specific bands were visualized using super signal west pico chemiluminescent substrate kit (Pierce Biotechnology, Rockford IL, U.S.A.) as described earlier (Marutha Ravindran and Ticku, 2006a, 2006b; Marutha Ravindran et al., 2007). Association pattern of the δ -subunit with other subunits of GABA_A receptors was also investigated in a reversible manner, i.e., 1000 μ g of the immunoprecipitated proteins of various subunits (α_1 , α_4 , α_6 , $\beta_2/3$, or γ_2) of the GABA_A receptors were transferred to the membrane and were incubated with primary antibody for the δ -subunit of the GABA_A receptors and then with the peroxidase-coupled secondary antibody as detailed earlier. Negative control experiments were performed in parallel as described above.

4.2. Radioligand binding to the brain membrane homogenate

Membranes from the rat cerebral cortex and cerebellum were prepared as described previously (Mehta and Ticku, 1998). Briefly, the frozen tissue was thawed and homogenized in ice-cold 0.32 M sucrose, pH 7.4 (20 ml/g of tissue). The mixture was then centrifuged at 1000 g for 10 min at 4°C so as to discard the P₁ fraction. This step was omitted in the case of cerebellum. The supernatant or the homogenized tissue was then centrifuged at 140,000 g for 30 min at 4°C to obtain the mitochondrial plus microsomal fraction. This fraction was dispersed in ice-cold purified water, and homogenized by a Brinkman Polytron at a setting of six for two 10-sec bursts, 10 sec apart. The suspension was centrifuged at 140,000 g for 30 min at 4°C. The pellet was then resuspended in ice-cold Tris-HCl buffer (50 mM, pH 7.4), and centrifuged at 140,000 g for 30 min at 4°C. The pellet was resuspended in Tris-HCl buffer, and kept frozen overnight at –80°C. After thawing, Tris-HCl buffer was added to the tissue, and the mixture was centrifuged at 140,000 g for 30 min at 4°C three times. The membranes were then suspended in Tris-HCl (50 mM, pH 7.4), distributed in aliquots, and kept frozen at –80°C until use. On the day of assay, the tissue was thawed and washed one time with buffer as before (140,000 g for 30 min at 4°C) and then resuspended in assay buffer. [³H]Muscimol and [³H]Ro 15–4513 binding were measured using a filtration method as described previously (Mehta and Shank, 1995; Mehta and Ticku, 1998). Briefly, aliquots (0.1–0.2 mg protein) of membrane preparation

in Tris-HCl buffer (50 mM, pH 7.4) were incubated with either [^{3} H]Ro 15–4513 (2 nM) at 4°C for 90 min or [^{3} H]muscimol (3 nM) at 4°C for 45 min in the absence and presence of different concentrations of ethanol. In the case of [^{3} H]Ro 15–4513 binding, all the assay tubes also contained diazepam (10 μ M) so as to displace the diazepam-sensitive binding. Non-specific binding was determined using Ro 15–4513 (10 μ M) or GABA (100 μ M) for [^{3} H]Ro 15–4513 and [^{3} H]muscimol binding, respectively. Radioligand binding to the GABA_A receptors was terminated by separating the membrane material from the incubation medium by vacuum filtration membrane harvester (Brandel MB-48L membrane harvester). The samples were washed twice with 2 ml of ice-cold Tris-HCl buffer (50 mM, pH 7.4). Radioactivity was quantified by liquid scintillation spectrometry. The data are expressed as mean $\pm$ S.E.M.

4.3. Radioligand binding to the immunoprecipitated receptor assemblies

Membranes from the rat cerebellum and hippocampus were prepared as described above (Mehta and Ticku, 1998). The GABA_A receptors solubilization, immunoprecipitation and [^{3} H]muscimol binding assays were performed as described by us earlier (Mehta and Ticku, 1999b, 2005). Briefly, GABA_A receptors were solubilized in modified radioimmune precipitation assay buffer (RIPA), i.e., solubilization buffer (pH 7.4) containing sodium chloride (0.137 M), sodium deoxycholate (1% w/v), Triton X-100 (1% v/v), sodium dodecyl sulfate, i.e., SDS (0.1% w/v), Tris (10 mM) and a cocktail of protease inhibitors containing EDTA (1 mM), EGTA (1 mM), benzamidine HCl (2 mM), trypsin inhibitor type 1-S (0.1 mg/ml), bacitracin (0.1 mg/ml) and phenylmethylsulfonyl fluoride (0.3 mM). After incubation for 1 h at 4°C, insoluble material was removed by centrifugation (100,000 g for 1 h at 4°C). A sample of 400 μ l ($\approx$ 300 μ g protein) of the solubilized receptors was incubated overnight at 4°C with 30 μ l of the antiserum for the rat GABA_A receptors δ -subunit. The receptor-antibody complexes were recovered by incubation with protein A-agarose suspension (60 μ l of 40% v/v) followed by centrifugation. Immunoprecipitation was quantified by determining the binding of [^{3} H]muscimol (15 nM) to the immunoprecipitated pellet and supernatant. Non-specific radioligand binding was determined with GABA (100 μ M). The data are expressed as mean $\pm$ S.E.M.

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References

- Araujo F, Ruano D, Vitorica J. Absence of association between δ and γ_2 subunits in native GABA_A receptors from rat brain. *Eur J Pharmacol* 1998;347:347–353. [PubMed: 9653902]
- Benke D, Mertens S, Trzeciak A, Gillissen D, Mohler H. GABA_A receptors display association of γ_2 -subunit with α_1 - and $\beta_{2/3}$ -subunits. *J Biol Chem* 1991;266:4478–4483. [PubMed: 1847922]
- Bianchi MT, Haas KF, Macdonald RL. Alpha1 and alpha6 subunits specify distinct desensitization, deactivation and neurosteroid modulation of GABA_A receptors containing the delta subunit. *Neuropharmacology* 2002;43:492–502. [PubMed: 12367596]
- Borghese CM, Storustovu SI, Ebert B, Herd MB, Belelli D, Lambert JJ, Marshall G, Wafford KA, Harris RA. The δ subunit of γ -aminobutyric acid type A receptors does not confer sensitivity to low concentrations of ethanol. *J Pharmacol Exp Ther* 2006;316:1360–1368. [PubMed: 16272217]
- Casagrande S, Cupello A, Pellistri F, Robello M. Only high concentrations of ethanol affect GABA_A receptors of rat cerebellum granule cells in culture. *Neurosci Lett* 2007;414:273–276. [PubMed: 17234340]
- De Blas AL. Brain GABA_A receptors studied with subunit-specific antibodies. *Mol Neurobiol* 1996;12:55–71. [PubMed: 8732540]
- Fritschy JM, Benke D, Mertens S, Oertel WH, Bachi T, Mohler H. Five subtypes of type A γ -aminobutyric acid receptors identified in neurons by double and triple immunofluorescence staining with subunit-specific antibodies. *Proc Natl Acad Sci USA* 1992;89:6726–6730. [PubMed: 1323116]

- Glykys J, Peng Z, Chandra D, Homanics GE, Houser CR, Mody I. A new naturally occurring GABA_A receptor subunit partnership with high sensitivity to ethanol. *Nat Neurosci* 2007;10:40–48. [PubMed: 17159992]
- Hanchar HJ, Wallner M, Olsen RW. Alcohol effects on γ -aminobutyric acid type A receptors: are extrasynaptic receptors the answer? *Life Sci* 2004;76:1–8. [PubMed: 15501475]
- Hanchar HJ, Dodson PD, Olsen RW, Otis TS, Wallner M. Alcohol-induced motor impairment caused by increased extrasynaptic GABA_A receptor activity. *Nat Neurosci* 2005;8:339–345. [PubMed: 15696164]
- Hanchar HJ, Chutsrinopkun P, Meera P, Supavilai P, Sieghart W, Wallner M, Olsen RW. Ethanol potently and competitively inhibits binding of the alcohol antagonist Ro 15–4513 to $\alpha_4/\beta_3\delta$ GABA_A receptors. *Proc Natl Acad Sci USA* 2006;103:8546–8551. [PubMed: 16581914]
- Jones A, Korpi ER, McKernan RM. Ligand-gated ion channel subunit partnerships: GABA_A receptor α_6 subunit gene inactivation inhibits δ subunit expression. *J Neurosci* 1997;17:1350–1362. [PubMed: 9006978]
- Kanakura Y, Druker B, DiCarlo J, Cannistra SA, Griffin JD. Phorbol 12-myristate 13-acetate inhibits granulocyte-macrophage colony stimulating factor-induced protein tyrosine phosphorylation in a human factor-dependent hematopoietic cell line. *J Biol Chem* 1991;266:490–495. [PubMed: 1845978]
- Marutha Ravindran CR, Ticku MK. Tyrosine kinase phosphorylation of GABA_A receptor subunits following chronic ethanol exposure of cultured cortical neurons of mice. *Brain Res* 2006a;1086:35–41. [PubMed: 16580644]
- Marutha Ravindran CR, Ticku MK. Tyrosine kinase phosphorylation of GABA_A receptor α_1 , β_2 , and γ_2 subunits following chronic intermittent ethanol (CIE) exposure of cultured cortical neurons of mice. *Neurochem Res* 2006b;31:1111–1118.
- Marutha Ravindran CR, Mehta AK, Ticku MK. Effect of chronic administration of ethanol on the regulation of tyrosine kinase phosphorylation of the GABA_A receptor subunits in the rat brain. *Neurochem Res* 2007;32:1179–1187. [PubMed: 17404840]
- Mehta AK, Shank RP. Interaction of abecarnil, bretazenil, and RO 19–8022 with diazepam-sensitive and -insensitive benzodiazepine sites in the rat cerebellum and cerebral cortex. *Life Sci* 1995;57:2215–2222. [PubMed: 7475974]
- Mehta AK, Ticku MK. Chronic ethanol administration alters the modulatory effect of 5 α -pregnan-3 α -ol-20-one on the binding characteristics of various radioligands of GABA_A receptors. *Brain Res* 1998;805:88–94. [PubMed: 9733930]
- Mehta AK, Ticku MK. An update on GABA_A receptors. *Brain Res Rev* 1999a;29:196–217. [PubMed: 10209232]
- Mehta AK, Ticku MK. Prevalence of the GABA_A receptor assemblies containing α_1 -subunit in the rat cerebellum and cerebral cortex as determined by immunoprecipitation: lack of modulation by chronic ethanol administration. *Mol Brain Res* 1999b;67:194–199. [PubMed: 10101248]
- Mehta AK, Ticku MK. Effect of chronic administration of ethanol on GABA_A receptor assemblies derived from α_2 -, α_3 -, β_2 - and γ_2 -subunits in the rat cerebral cortex. *Brain Res* 2005;1031:134–137. [PubMed: 15621022]
- Mertens S, Benke D, Mohler H. GABA_A receptor populations with novel subunit combinations and drug binding profiles identified in brain by α_5 - and δ -subunit-specific immunopurification. *J Biol Chem* 1993;268:5965–5973. [PubMed: 8383681]
- Mihalek RM, Bowers BJ, Wehner JM, Kralic JE, VanDoren MJ, Morrow AL, Homanics GE. GABA_A-receptor δ subunit knockout mice have multiple defects in behavioral responses to ethanol. *Alcohol Clin Exp Res* 2001;25:1708–1718. [PubMed: 11781502]
- Poltl A, Hauer B, Fuchs K, Tretter V, Sieghart W. Subunit composition and quantitative importance of GABA_A receptor subtypes in the cerebellum of mouse and rat. *J Neurochem* 2003;87:1444–1455. [PubMed: 14713300]
- Quirk K, Gillard NP, Ragan CI, Whiting PJ, McKernan RM. Model of subunit composition of γ -aminobutyric acid_A receptor subtypes expressed in rat cerebellum with respect to their α and γ/δ subunits. *J Biol Chem* 1994;269:16020–16028. [PubMed: 8206899]

- Quirk K, Whiting PJ, Ragan CI, McKernan RM. Characterisation of delta-subunit containing GABA_A receptors from rat brain. *Eur J Pharmacol* 1995;290:175–181. [PubMed: 7589211]
- Saxena NC, Macdonald RL. Assembly of GABA_A receptor subunits: role of the delta subunit. *J Neurosci* 1994;14:7077–7086. [PubMed: 7525894]
- Saxena NC, Macdonald RL. Properties of putative cerebellar gamma-aminobutyric acid A receptor isoforms. *Mol Pharmacol* 1996;49:567–579. [PubMed: 8643098]
- Shannon EE, Shelton KL, Vivian JA, Yount I, Morgan AR, Homanics GE, Grant KA. Discriminative stimulus effects of ethanol in mice lacking the γ -aminobutyric acid type A receptor δ subunit. *Alcohol Clin Exp Res* 2004;28:906–913. [PubMed: 15201633]
- Shivers BD, Killisch I, Sprengel R, Sontheimer H, Kohler M, Schofield PR, Seeburg PH. Two novel GABA_A receptor subunits exist in distinct neuronal subpopulations. *Neuron* 1989;3:327–337. [PubMed: 2561970]
- Storustovu S, Ebert B. Pharmacological characterization of agonists at δ -containing GABA_A receptors: functional selectivity for extrasynaptic receptors is dependent on the absence of γ_2 . *J Pharmacol Exp Ther* 2006;316:1351–1359. [PubMed: 16272218]
- Sundstrom-Poromaa I, Smith DH, Gong QH, Sabado TN, Li X, Light A, Wiedmann M, Williams K, Smith SS. Hormonally regulated $\alpha_4\beta_2\delta$ GABA_A receptors are a target for alcohol. *Nat Neurosci* 2002;5:721–722. [PubMed: 12118257]
- Sur C, Farrar SJ, Kerby J, Whiting PJ, Atack JR, McKernan RM. Preferential coassembly of α_4 and δ subunits of the γ -aminobutyric acid_A receptor in rat thalamus. *Mol Pharmacol* 1999;56:110–115. [PubMed: 10385690]
- Wallner M, Hancher HJ, Olsen RW. Ethanol enhances $\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$ γ -aminobutyric acid type A receptors at low concentrations known to affect humans. *Proc Natl Acad Sci USA* 2003;100:15218–15223. [PubMed: 14625373]
- Wei W, Faria LC, Mody I. Low ethanol concentrations selectively augment the tonic inhibition mediated by δ subunit-containing GABA_A receptors in hippocampal neurons. *J Neurosci* 2004;24:8379–8382. [PubMed: 15385620]
- Whiting PJ. GABA_A receptor subtypes in the brain: a paradigm for CNS drug discovery? *Drug Discov Today* 2003;8:445–450. [PubMed: 12801796]
- Wisden W, Laurie DJ, Monyer H, Seeburg PH. The distribution of 13 GABA_A receptor subunit mRNAs in the rat brain. I Telencephalon, diencephalons, mesencephalon. *J Neurosci* 1992;12:1040–1062. [PubMed: 1312131]
- Wohlfarth KM, Bianchi MT, Macdonald RL. Enhanced neurosteroid potentiation of ternary GABA_A receptors containing the delta subunit. *J Neurosci* 2002;22:1541–1549. [PubMed: 11880484]
- Yamashita M, Marszalec W, Yeh JZ, Narahashi T. Effects of ethanol on tonic GABA currents in cerebellar granule cells and mammalian cells recombinantly expressing GABA_A receptors. *J Pharmacol Exp Ther* 2006;319:431–438. [PubMed: 16844844]

Abbreviations

PMSF	phenylmethylsulfonyl fluoride
PBS	phosphate-buffered saline
SDS	sodium dodecyl sulfate

Fig 1.

Immunoprecipitation, dot blotting and immunostaining using various amounts of the tissue protein (1500 μ g, 1000 μ g, 500 μ g and 100 μ g) and antibody for the (a) α_1 -, (b) α_4 -, (c) α_6 -, (d) β_2 -, (e) β_3 -, (f) γ_2 -, or (g) δ -subunit of GABA_A receptors. For negative control (NC), affinity-purified non-immune rabbit polyclonal IgG was used. Lane 1: 1500 μ g; lane 2: 1000 μ g; lane 3: 500 μ g and lane 4: 100 μ g of the immunoprecipitated protein from the rat cerebellum (α_1 -, α_6 -, β_2 -, β_3 -, and γ_2 -subunits) or hippocampus (α_4 -subunit). Each experiment was performed three times.

Fig 2.

Co-localization analysis of δ -subunit with other subunits of GABA_A receptors in the rat cerebellum (α_1 -, α_6 -, β_2 -, β_3 -, and γ_2 -subunits) and hippocampus (α_4 -subunit) using immunoprecipitation and Western blotting techniques. Information regarding the use of various antibodies for immunoprecipitation is provided at the top of each strip, whereas the information regarding the use of antibodies for detection of the protein band in Western blot analysis is provided at the bottom of each strip. For negative control, affinity-purified non-immune rabbit polyclonal IgG (NC) and/or polypeptide for the GABA_A receptors δ -subunit antibody (20 μ g/ml; C) was used. Each experiment was performed three times.

Table 1

Effect of ethanol on [^3H]muscimol (3 nM) binding and diazepam-insensitive [^3H]Ro 15–4513 (2 nM) binding in the membranes from the rat cerebellum and cerebral cortex.

Each value is mean $\pm$ S.E.M. of number of individual experiments indicated (n), each performed in triplicate.

Group	% Inhibition of binding [^3H]Muscimol (n)	[^3H]Ro 15–4513 (n)
<i>Cerebellum</i>		
Ethanol (1–50 mM)	< 5.0 (8)	< 5.0 (4)
Ethanol (100 mM)	2.1 $\pm$ 1.6 (6)	5.4 $\pm$ 2.3 (4)
Ethanol (200 mM)	5.6 $\pm$ 1.1 (6)	6.3 $\pm$ 2.1 (5)
Ethanol (500 mM)	6.7 $\pm$ 0.9 (6)	17.8 $\pm$ 0.8 (5)
<i>Cerebral cortex</i>		
Ethanol (1–50 mM)	< 5.0 (7)	< 5.0 (5)
Ethanol (100 mM)	5.4 $\pm$ 2.5 (6)	7.9 $\pm$ 0.8 (5)
Ethanol (200 mM)	6.6 $\pm$ 1.9 (7)	10.4 $\pm$ 0.7 (5)
Ethanol (500 mM)	10.1 $\pm$ 2.4 (7)	21.9 $\pm$ 1.2 (5)

Basal [^3H]muscimol (3 nM) binding was found to be 1.38 $\pm$ 0.08 pmol/mg protein and 0.39 $\pm$ 0.05 pmol/mg protein in the membranes from the rat cerebellum and cerebral cortex, respectively. Basal [^3H]Ro 15–4513 (2 nM) diazepam-insensitive binding was found to be 0.32 $\pm$ 0.02 pmol/mg protein and 0.027 $\pm$ 0.002 pmol/mg protein in the membranes from the rat cerebellum and cerebral cortex, respectively.

Table 2

Effect of ethanol on [^3H]muscimol (15 nM) binding to the immunoprecipitated δ -subunit-containing GABA $_A$ receptor assemblies in the rat cerebellum and hippocampus.

Each value is mean $\pm$ S.E.M. of three individual experiments, each performed in triplicate.

Treatment	% Inhibition of binding Cerebellum	Hippocampus
Ethanol (5–50 mM)	< 5.0	<5.0
Ethanol (200 mM)	7.8 $\pm$ 0.8	10.5 $\pm$ 1.7
Ethanol (500 mM)	12.4 $\pm$ 0.9	13.9 $\pm$ 2.1

[^3H]muscimol (15 nM) binding to the solubilized GABA $_A$ receptors from the rat cerebellum and hippocampus was found to be 0.12 $\pm$ 0.01 pmol/mg protein (n=3) and 0.09 $\pm$ 0.01 pmol/mg protein (n=3), respectively.