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

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γ-Aminobutyric acid type A receptors (GABARs) have long been implicated in mediating ethanol (EtOH) actions, but so far most of the reported recombinant GABAR combinations have shown EtOH responses only at fairly high concentrations (≥60 mM). We show that GABARs containing the δ-subunit, which are highly sensitive to γ-aminobutyric acid, slowly inactivating, and thought to be located outside of synapses, are enhanced by EtOH at concentrations that are reached with moderate, social EtOH consumption. Reproducible ethanol enhancements occur at 3 mM, a concentration six times lower than the legal blood-alcohol intoxication (driving) limit in most states (0.08% wt/vol or 17.4 mM). GABARs responsive to these low EtOH concentrations require the GABAR δ-subunit, which is thought to be associated exclusively with α_4- and α_6-subunits *in vivo*, and the β_3-subunit, which has recently been shown to be essential for the *in vivo* anesthetic actions of etomidate and propofol. GABARs containing β_2- instead of β_3-subunits in $\alpha_4\beta\delta$- and $\alpha_6\beta\delta$-receptor combinations are almost 10 times less sensitive to EtOH, with threshold enhancement at 30 mM. GABARs containing γ_2- instead of δ-subunits with $\alpha_4\beta$ and $\alpha_6\beta$ are three times less sensitive to EtOH, with threshold responses at 100 mM, a concentration not usually reached with social EtOH consumption. These combined findings suggest that ''extrasynaptic'' δ-subunit-containing GABARs, but not their ''synaptic'' γ-subunit-containing counterparts, are primary targets for EtOH.

Despite the fact that ethanol (EtOH) is the most widely used psychoactive agent, its actions on brain functions are poorly understood. Several types of receptors and channels have been shown to be functionally altered by EtOH, which include *N*-methyl-D-aspartate (1) and non-*N*-methyl-D-aspartate glutamate receptors (2, 3), serotonin (4), glycine (5, 6), and GABARs (7, 8), and G protein-coupled inwardly rectifying K^+ channels (9, 10). With a few exceptions (3, 8–12), EtOH effects on these targets are seen only at fairly high concentrations (≥60 mM).

The GABAR, the major inhibitory neurotransmitter receptor, has been a long-time focus for studies on EtOH and anesthetic actions. For example, it has been shown that EtOH at low intoxicating concentrations was able to enhance Cl^- flux in synaptoneurosomes (13, 14) and cultured neurons (15). However, electrophysiological studies of GABARs in single neurons and recombinant receptors showed current enhancement only at fairly high concentrations (>50 mM) of EtOH (5, 7, 16), which now appears to be due to the fact that these studies focused on synaptic and/or γ-subunit-containing receptors. It is thought that replacement of the γ-subunit in the GABAR 2α–2β–1γ pentameric complex by the δ-subunit changes not only the localization of the receptor from mainly postsynaptic to extrasynaptic, but also leads to up to a 50-fold increase in γ-aminobutyric acid (GABA) affinity and slower desensitization (17–19). These functional properties are consistent with $\alpha\beta\delta$ GABARs, which are activated by ambient extracellular GABA concentrations (thought to be on the order of 0.5–1 μM; ref. 20). The tonic currents flowing through these channels contrast with synaptic $\alpha\beta\gamma$ GABARs, which open only briefly (≈10 ms) in response to near-saturating amounts (≥1 mM peak concentration) of GABA released into the synaptic cleft.

Even though δ-subunits can be forced to form receptors with all α- and β-subunits tested in recombinant systems (17, 18), *in vivo* they appear to associate virtually exclusively with α_4- (21) and α_6-subunits (22). The α_6-subunit protein is expressed only in cerebellar granule cells, whereas α_4-subunits have a more widespread distribution and are expressed (with decreasing abundance) in the thalamus, the dentate gyrus, the striatum, the outer layers of the cortex, and at lower levels in other brain areas like the hippocampus. In cerebellar granule cells, the δ-subunit together with the α_6-subunit is exclusively extrasynaptic (23); and, in neurons expressing the α_4/δ combination, an extrasynaptic location also seems likely (24). Consistent with its exclusive association with α_4- and α_6-subunits, the distribution of the δ-subunit revealed by immunostaining has a striking resemblance with α_4-immunoreactivity in mouse brain (25), except for the cerebellar granule cell layer where the closely related α_6-subunit replaces α_4.

In chronic intermittent EtOH-treated rats, a model for human alcohol-withdrawal syndrome, δ-subunit protein levels decrease in the hippocampus, whereas α_4- and γ_2-proteins increase. The changes in synaptic benzodiazepine pharmacology suggest that α_4- replaces α_1-subunits in synaptic $\alpha\beta\gamma$-receptors in chronic intermittent EtOH-treated rats (26). A comparison of $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_2$ GABARs studied in expression systems revealed that the δ-subunit-containing receptors showed greater enhancement by etomidate, pentobarbital, propofol, and steroids [tetrahydrodeoxycorticosterone (THDOC) and alphaxalone] than those containing the γ_2-subunit (18, 19, 27, 28). In knock-in mice, the *in vivo* actions of etomidate and propofol were almost completely abolished by a point mutation (N256M) in the GABAR β_3-subunit, which demonstrates that GABARs containing the β_3-subunit mediate the *in vivo* effects of these general anesthetics (29). In contrast, mice containing the etomidate-insensitive N265S mutation in the β_2-subunit lose sedative but not the anesthetic effects of etomidate (30).

Here we show that recombinant $\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$ GABARs are uniquely sensitive to ethanol, with a dose–response relationship mirroring the well known effects of alcohol consumption on the human brain. Surprisingly, ethanol was much more effective on β_3- than on β_2-containing $\alpha_4\beta\delta$- and $\alpha_6\beta\delta$-receptors, which demonstrates that the incorporation of the GABAR β_2- or β_3-subunits can lead to functionally distinct receptors in recom-

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Abbreviations: THDOC, tetrahydrodeoxycorticosterone; GABA, γ-aminobutyric acid; GABAR, GABA type A receptor; EtOH, ethanol; IPSP/C, inhibitory postsynaptic potential/inhibitory postsynaptic current.

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binant expression systems. In fact, the EtOH sensitivity was increased only 3-fold by replacing the γ_2- with the δ-subunit, whereas an almost 10-fold increase was observed by replacement of β_2 with β_3 in the $\alpha\beta\delta$ GABARs. These findings lead us to propose that extrasynaptic $\alpha_4\beta_3\delta$- or $\alpha_6\beta_3\delta$-subunit-containing GABARs are primary targets for EtOH. This hypothesis probably applies also for other GABAR-specific general anesthetics (etomidate, propofol, and steroid anesthetics). The finding that these subtypes of GABAR are likely targets of EtOH action is consistent with their anatomical distribution in brain regions that mediate EtOH effects on behavior, such as the cerebellum (motor coordination), the hippocampal formation (amnesic effects), and the thalamus (sleep-promoting and possibly anesthetic effects).

Methods

cRNA. Rat GABAR subunit cDNAs were obtained from A. Tobin (University of California, Los Angeles; α_1), H. Luddens (University of Mainz, Mainz, Germany; α_4), R. Macdonald (Vanderbilt University, Nashville, TN; α_6, δ), D. Pritchett (University of Pennsylvania, Philadelphia; β_2), L. Mahan (National Institutes of Health, Bethesda; β_3), and D. S. Weiss (University of Alabama, Birmingham; γ_{2L} and γ_{2S}). The ORFs and part of the 3′ UTRs of the rat α_4- and δ-subunits were amplified by PCR and cloned into a vector containing the 5′ UTR of the Shaker potassium channel. The coding regions of all GABAR cDNA clones used in this study were verified by fluorescent (BigDye3, Sigma-Aldrich) sequencing. mRNA was transcribed from linearized template plasmids by using the mMESSAGE mMACHINE kits (Ambion, Austin, TX). We purified cRNA transcripts by LiCl precipitation and analyzed transcript quality and concentration by photometry and gel electrophoresis. Oocytes were injected with 0.4 ng of α- and β-subunit cRNA and 2 ng (in some cases, even 4 ng) of δ- or γ_2-cRNA. This 5- or 10-fold excess of δ- and γ-cRNA over α and β was used to avoid "contamination" by functional α/β-subunit receptors. Currents were measured 3–4 days after oocyte injection for γ-subunit-containing receptors, whereas oocytes injected with δ-subunit-containing receptors, because of their apparently low expression levels, had their currents measured 7–8 days after injection.

Electrophysiology. We measured GABAR currents in oocytes with an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA) in the two-electrode voltage-clamp configuration. Electrodes were filled with 3 M KCl and had resistances between 0.5 and 1.5 MΩ, when measured dipped in the bath solution. The oocyte chamber was continuously perfused with ND96 bath solution (composition, 96 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes, pH 7.5) with drugs and treatments mentioned. Solution exchanges were triggered with a programmable valve bank switching a three-way solenoid valve, and bath-volume exchange times were in the range of 1–3 s. Currents were measured at a holding potential (V_H) of −80 mV (unless indicated otherwise), where GABA applications evoke an inward current in oocytes. Currents and voltages were recorded on a two-channel chart recorder (Soltec 1242, Sun Valley, CA). To minimize voltage-clamp errors, only current responses of <1 μA were considered for analysis. Chart recordings were digitized by using GRAPH DIGITIZATION software (N. Rodionov). GABA, EtOH, and the steroid THDOC (5β-pregnane-3α,21-diol-20-one) were obtained from Sigma. THDOC was dissolved in DMSO (10 mM stock solution). Etomidate was the clinical formulation from Bedford Laboratories (Bedford, OH). The curve fits for the GABA dose–responses were generated by the nonlinear sigmoidal dose–response equation $I/I_{max} = 1/[1 + [EC_{50}/(GABA)^n]$, where EC_{50} is the concentration of drug eliciting a half-maximal response, n is the Hill coefficient, I_{max} is the maximum current, and I is the GABA-evoked current. Values for EC_{50} and percent EtOH enhancements were calculated for individual cells and combined to give means with 95% confidence intervals.

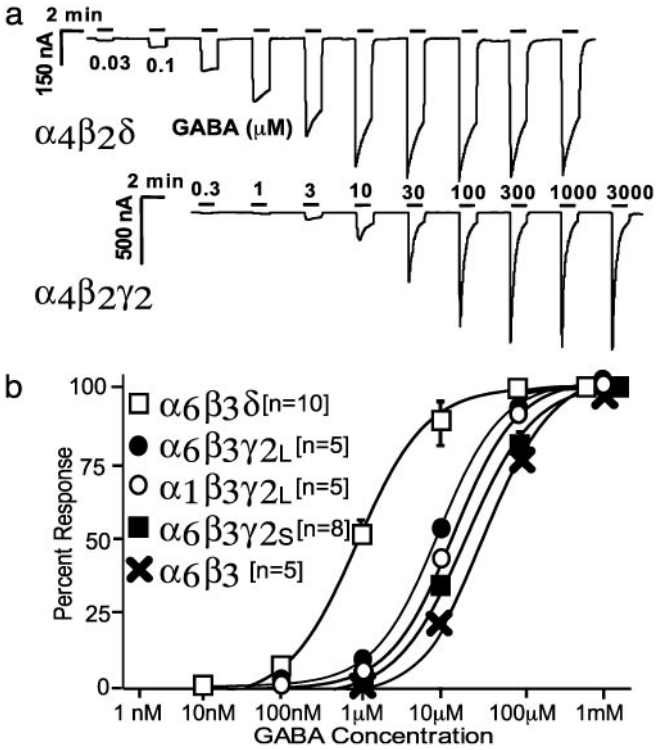

Fig. 1. Comparison of synaptic ($\alpha\beta\gamma$) and extrasynaptic ($\alpha\beta\delta$) GABARs. (*a*) Comparison of GABAR currents expressed in *Xenopus* oocytes. GABARs composed of $\alpha_4\beta_2\delta$-subunits show a slower desensitization and are activated at much lower concentrations of GABA than those formed by $\alpha_4\beta_2\gamma_{2S}$. (*b*) GABA dose– responses on GABAR subunit combinations containing either α_4- or α_6-subunits, with β_2- or β_3-subunits, with and without γ_{2S}-, γ_{2L}-, or δ-subunits. The plots shown are for $\alpha_6\beta_3\delta$, $\alpha_6\beta_3\gamma_{2L}$, $\alpha_1\beta_3\gamma_{2L}$, $\alpha_6\beta_3\gamma_{2S}$, and α6β3. GABARs containing α_4- in place of α_6-, and β_2- instead of β_3-subunits are virtually identical (see Table 1 for EC_{50} values). The $\alpha\beta$-subunits produce small-current levels and have a fairly low potency for GABA ($EC_{50} \approx 22$ μM GABA). γ-Subunit-containing receptors respond to lower concentrations of GABA (γ_{2S}, $EC_{50} \approx 18$ μM GABA; $\alpha_1\beta_3\gamma_{2L}$, $EC_{50} \approx 10$ μM GABA; $\alpha_6\beta_3\gamma_{2L}$, ≈ 8 μM GABA). δ-containing receptors are the most GABA-sensitive with just-detectable responses evident at concentrations as low as 30 nM GABA ($EC_{50} \approx 0.57$ μM GABA).

Results

Expression and Functional Properties of $\alpha_4\beta_3\delta$ or $\alpha_6\beta_3\delta$ Receptors in *Xenopus* Oocytes. Both GABAR α_4- and δ-subunits have been reported to be difficult to express in recombinant systems (27, 31). In most published studies, the α_4-subunit and, in some cases, also the δ-subunit cDNAs (19) were used as chimeras in which the signal sequences and the 5′ UTRs were replaced by the signal sequence and the 5′ UTRs of the bovine α_1 GABAR (19, 27, 28, 31). To prevent expression problems due to inhibitory 5′ UTRs, we amplified the coding regions of α_4- and δ-subunits by PCR and cloned them into an expression vector, thereby replacing upstream noncoding parts of these clones. Rat α_4- or α_6-subunits were expressed together with rat β_2- or β_3- and δ- or γ_{2S} (or γ_{2L})-subunits in *Xenopus* oocytes. The functional properties of δ-subunit-containing receptors confirmed previous findings obtained with recombinant receptors in eukaryotic cells (17–19, 27) that δ-subunit-containing recombinant receptors were >20 times more sensitive to GABA (than corresponding γ_{2S}-subunit-containing receptors) and showed slower desensitization. These properties are consistent with their proposed function as extra-

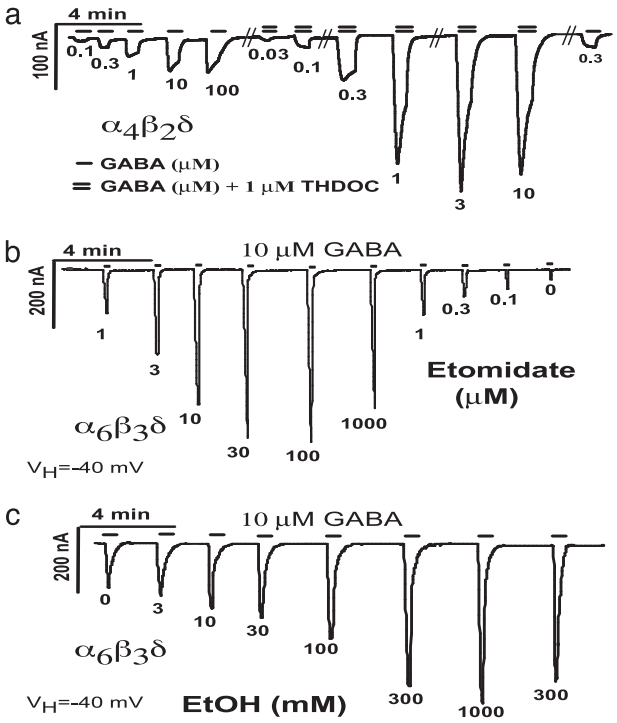

Fig. 2. δ-Subunit-containing GABARs as targets for neuroactive steroids, the general anesthetic etomidate, and ethanol. (*a*) GABA dose–response curve of $\alpha_4\beta_2\delta$ GABAR alone and in the presence of 1 μM THDOC shows an up to 5-fold increase in peak currents with 1 μM THDOC even at saturating GABA concentrations. (*b*) Dose–response curve of the general anesthetic etomidate on $\alpha_6\beta_3\delta$ GABAR current. Etomidate was coapplied with almost saturating amounts of GABA (10 μM = EC_{95}). (*c*) EtOH coapplication with 10 μM (EC_{95}) GABA causes an increase in current levels in $\alpha6\beta3\delta$ GABARs as low as 3 mM and triples the peak currents at 1 M. Shown are single traces with full dose–response curves that demonstrate that GABA is only a partial agonist at δ-subunit-containing receptors. Similar results have been obtained with application of selected concentrations of THDOC, etomidate, and EtOH and with other δ-subunit-containing GABARs. Peaks marked with "0" show current responses to 10 μM GABA without etomidate or EtOH. Application of 1 μM THDOC, 300 μM etomidate, or 1 M EtOH alone does not evoke significant currents in $\alpha_6\beta_3\delta$-expressing oocytes (not shown).

synaptic receptors mediating tonic inhibition (20). Recordings that illustrate the functional differences between receptors containing δ-subunits versus those containing γ_{2S}-subunits are shown in Fig. 1*a*. GABA dose–response curves of $\alpha\beta\gamma$ and $\alpha\beta\delta$ and binary $\alpha\beta$ GABARs are shown in Fig. 1*b*. No significant differences occurred in the GABA dose–response curves when α_4 was replaced by α_6 or when β_2 was replaced by β_3 in $\alpha\beta\gamma_2$ ($EC_{50} \approx 18$ μM), $\alpha\beta\delta$ ($EC_{50} \approx 0.57$ μM), or $\alpha\beta$ ($EC_{50} \approx 22$ μM) subunit combinations. The fact that receptors containing α_4-subunits with a modified 5′-UTR expressed current levels similar to those containing α_6-subunits suggests that the 5′ untranslated region in the "native" α_4-subunit is responsible for the reported poor expression in recombinant expression systems rather than inefficient processing of the native α_4-subunit protein.

GABARs Containing the δ-Subunit at Saturating GABA Concentrations: GABA as a Partial Agonist. In all receptor combinations tested, GABA-evoked peak currents from γ_{2S}-containing receptors were much larger than those from corresponding δ-subunit-containing receptors when measured at the same time after injection. This finding held true despite our efforts to improve expression by modifying the 5′ untranslated region in the δ-subunit cDNA. However, smaller currents are not due to the fewer channels expressed, because coapplication of a high concentration (1 μM) of the neurosteroid THDOC together with GABA increased current levels in $\alpha_4\beta_2\delta$-receptors about 5-fold at all GABA concentrations tested (Fig. 2), consistent with the reported effects of THDOC on $\alpha_6\beta_3\delta$ (18) and $\alpha_4\beta_3\delta$ (19). Because the efficacy of etomidate is much greater at $\alpha_4\beta_3\delta$ versus $\alpha_4\beta_3\gamma_2$ GABARs (19), we tested whether etomidate would increase peak current responses on $\alpha_6\beta_3\delta$ GABARs in oocytes. Etomidate (from 0.1 to 1000 μM) coapplied with a close to saturating concentration of GABA (10 μM or EC_{95}; see Fig. 1*a*) led to a dramatic dose-dependent enhancement of peak current responses (Fig. 2*b*). At the most effective concentration of etomidate (100 μM), peak current increases about 20-fold. This result is probably an underestimation because of the fairly slow perfusion around the oocyte, which tends to obscure peak current responses at desensitizing receptors. This finding is the likely explanation for the lower peak current responses with 10 μM GABA + 1,000 μM etomidate (Fig. 2*b*) and for the decrease in peak currents 10 μM GABA + 1 μM THDOC (Fig. 2*a*). This dramatic enhancement of peak GABA currents (even at saturating GABA concentrations) by etomidate and THDOC suggests that the low-current levels we observe in δ-subunit-containing receptors are caused by GABA being a partial agonist on $\alpha\beta\delta$ GABAR, rather than by a problem with expression (i.e., the number of functional channels formed).

Table 1. GABA EC_{50} values for all subunits studied

Receptor	EC_{50}, nM	*n*
$\alpha_6\beta_3\delta$	0.67 ± 0.03	10
$\alpha_4\beta_3\delta$	0.62 ± 0.02	9
$\alpha_6\beta_2\delta$	0.75 ± 0.08	9
$\alpha_4\beta_2\delta$	0.5 ± 0.07	7
$\alpha_6\beta_3\gamma_{2S}$	18.8 ± 0.03	8
$\alpha_6\beta_3\gamma_{2L}$	7.6 ± 0.6	5
$\alpha_4\beta_3\gamma_{2S}$	16.6 ± 0.7	6
$\alpha_4\beta_2\gamma_{2S}$	17.7 ± 0.6	6
$\alpha_1\beta_3\gamma_{2L}$	10.3 ± 0.4	5
$\alpha_6\beta_3$	21.6 ± 1.8	5
$\alpha_4\beta_3$	22.5 ± 2.3	4
$\alpha_6\beta_2$	21.4 ± 1.1	4
$\alpha_4\beta_2$	22.0 ± 2.7	4

Values were calculated for each individual cell and represent the arithmetic mean ± SEM from a number (*n*) of various cells.

$\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$ GABARs Show Threshold EtOH Enhancement at Concentrations Reached During Moderate Social Ethanol Consumption. Because EtOH is often classified as an anesthetic (32), and GABARs have long been implicated in mediating EtOH effects (7), we tested EtOH on δ-subunit-containing GABARs under the same conditions as etomidate (coapplication of EtOH with 10 μM GABA). With the $\alpha_6\beta_3\delta$ GABARs, we saw just-detectable increases at 3 mM and a 2-fold increase in peak currents at 1 M EtOH (Fig. 2*c*). In the range from 0 to 300 nM etomidate, the amount of peak current enhancement coincides with that seen with 0–300 mM EtOH at $\alpha_6\beta_3\delta$-subunit-containing GABARs (compare Fig. 2*b* with Fig. 2*c*).

To further characterize EtOH responses, we tested at a GABA concentration that produced 20% of peak currents (EC_{20}) for each subunit combination (300 nM for $\alpha\beta\delta$, 10 μM for $\alpha\beta\gamma$, and 30 μM for $\alpha\beta$ GABAR combinations; see Fig. 1*b*). Measuring at a nonsaturating EC_{20} GABA concentration is reasonable for extrasynaptic GABARs, because they operate at usually nonsaturating GABA concentrations.

EtOH effects were recorded by using short ethanol coappli-

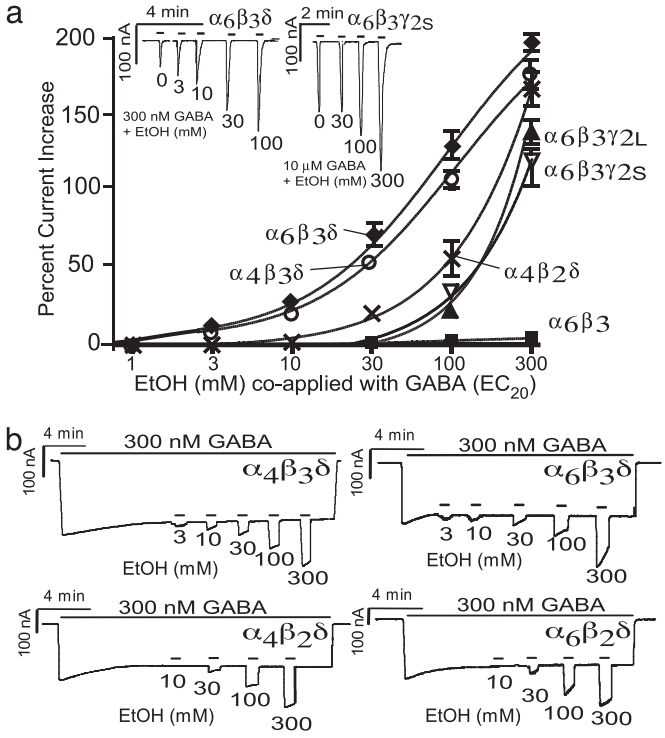

Fig. 3. Both the β_3- and δ-subunits are required for high ethanol sensitivity. (*a*) EtOH current enhancement when coapplied with respective EC_{20} values of the various subunit combinations. $\alpha\beta\delta$-Subunits show the largest enhancement, β_3-containing receptors being the most sensitive. γ-containing GABAR currents show significant potentiation only at 100 mM EtOH, and $\alpha\beta$-subunits seem to be completely insensitive. The plots shown are for $\alpha_6\beta_3\delta$ (◆), $\alpha_4\beta_3\delta$ (○), $\alpha_4\beta_2\delta$ (×), $\alpha_6\beta_3\gamma_{2L}$ (▲), $\alpha_6\beta_3\gamma_{2S}$ (▽), and $\alpha_6\beta_3$ (■) (n = 13, 12, 15, 10, 9, and 5, respectively); the remaining combinations with δ, γ_{2S}, or γ_{2L} or neither were virtually indistinguishable from the subunits represented (see Table 2 for pooled EtOH-response values). (*b*) EtOH effects on tonically activated receptors. Replacement of β_2- with β_3-subunits in $\alpha_4\beta\delta$ or $\alpha_6\beta\delta$ GABARs leads to an almost 10-fold increase in EtOH sensitivity. EtOH response (from 3 to 300 mM) on $\alpha_4\beta_2\delta$-, $\alpha_4\beta_3\delta$-, $\alpha_6\beta_2\delta$-, and $\alpha_6\beta_3\delta$-containing GABARs activated by steady-state 300 nM GABA ($\approx EC_{30}$).

cation (from 1 to 300 mM) with EC_{20} GABA concentrations to evoke a peak current, with recovery periods of at least 40 s between applications (Fig. 3 *Inset*). The peak responses were plotted as an increase over peak currents with EC_{20} GABA applications alone (Fig. 3*a*). GABARs composed of $\alpha_6\beta_3\delta$ and $\alpha_4\beta_3\delta$ showed the highest EtOH sensitivity and had a similar enhancement at low EtOH concentrations, whereas at concentrations of >10 mM EtOH, $\alpha_6\beta_3\delta$-receptors showed greater enhancement by EtOH than $\alpha_4\beta_3\delta$-receptors. Receptors containing the β_2-subunit were much less sensitive than the corresponding β_3-containing receptors at low EtOH concentrations [threshold responses at 30 mM (Fig. 3*a*)] but not at very high (300 mM) EtOH concentrations. Receptors containing the γ_2-subunit showed significant EtOH activation starting at a concentration of 100 mM, and GABARs composed of only $\alpha\beta$-subunits were almost completely insensitive, even at 300 mM EtOH (see Table 1 for percent EtOH increases for each subunit combination).

In addition to EtOH effects evaluated by GABA/EtOH coapplication, we used a protocol where we tried to mimic the physiological *modus operandi* of extrasynaptic GABA receptors (Fig. 3*b*). This protocol involved the prolonged application of 300 nM GABA (i.e., EC_{20}) to the oocyte, where currents relaxed to the steady-state activation level to which increasing concentrations of EtOH (in 300 nM GABA) were applied (Fig. 3*b*). Similar to results seen in the GABA-EtOH coapplication protocol, we saw the threshold current enhancement at 30 mM EtOH with $\alpha_4\beta_2\delta$, whereas with the $\alpha_6\beta_3\delta$ or $\alpha4\beta_3\delta$ combinations, the threshold response was observed at 3 mM, a concentration almost six times lower than the human blood-alcohol legal driving limit of 17.4 mM. Although the EtOH potency was similar under both measuring conditions, the absolute current enhancement by EtOH (efficacy) was about twice as large when GABA/EtOH was coapplied to nondesensitized receptors as in the EtOH application under steady-state conditions. This finding suggests that EtOH responsiveness may be diminished during the prolonged presence of GABA, possibly because of slowly populated states of the receptor with diminished EtOH sensitivity. It is interesting that EtOH responses in the presence of 300 nM GABA seem to have their own, only weakly dose-dependent, desensitization (see Fig. 3*b*).

Discussion

An Extrasynaptic GABAR Subunit Combination Is Highly Sensitive to Ethanol. EtOH has been shown to enhance γ-subunit-containing GABARs and glycine receptors in recombinant systems and in slice recordings to increase inhibitory postsynaptic potential/inhibitory postsynaptic current (IPSP/C) decay times. However, in most studies the concentrations needed (>40 mM) to show significant GABAR current enhancement are beyond the usual blood-ethanol concentrations reached during human alcohol consumption and would be potentially life-threatening (33). The median lethal blood-alcohol concentration in Finnish people was reported to be 0.33% or 72 mM (33). It seems unlikely, therefore, that synaptic receptors are primary EtOH responders, but they may contribute to EtOH toxicity at high concentrations. We confirm the relative EtOH insensitivity of γ-subunit-containing GABARs, which in our hands showed just-detectable EtOH enhancement at 100 mM but not at 30 mM, whereas GABARs containing the $\alpha_4\beta_2\delta$- or $\alpha_6\beta_2\delta$-subunits are about three times more sensitive. Most surprisingly, replacement of the β_2- with the β_3-subunit increased the EtOH sensitivity 10-fold, showing that β-subunit isoforms can lead to functional differences in recombinant GABARs. It is likely that the stimulation of GABA-

Table 2. Ethanol enhancement of GABAR

Receptors	*n*	3 mM EtOH	30 mM EtOH	100 mM EtOH	300 mM EtOH
$\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$	25	15.6 ± 0.8*	74.8 ± 1.5*	109.5 ± 1.9*	199.0 ± 2.6
$\alpha_4\beta_2\delta$ and $\alpha_6\beta_2\delta$	29	0	20.8 ± 2.7*	57.1 ± 7.0*	169.8 ± 5.1
$\alpha_1\beta_2\gamma_{2L}$	11	0	0	36.7 ± 3.4	150.1 ± 12.9
$\alpha_6\beta_3\gamma_{2L}$	10	0	0	35.7 ± 4.6	154.0 ± 11.0
$\alpha_4\beta_2\gamma_{2s}$, $\alpha_6\beta_2\gamma_{2s}$, $\alpha_4\beta_3\gamma_{2s}$, and $\alpha_6\beta_3\gamma_{2s}$	36	0	0	29.3 ± 2.3*	134.4 ± 3.9*
$\alpha_4\beta_2$, $\alpha_6\beta_2$, $\alpha_4\beta_3$, and $\alpha_6\beta_3$	21	0	0	2.4 ± 0.4	3.6 ± 0.3

Values are increase of GABA EC_{20} peak responses in percent ± SD at the indicated EtOH concentrations with different subunit combination. Values for α_4- and α_6-receptors were pooled, and all those for β_2- and β_3-receptors in $\alpha\beta\gamma$ and $\alpha\beta$ combinations. Asterisks indicate significant differences ($P < 0.005$); e.g., a significant difference occurs in EtOH enhancement between $\alpha\beta\gamma$ and $\alpha\beta$, but no significant difference ($P > 0.05$) was seen between $\alpha\beta2\delta$ and $\alpha\beta\gamma$ (Student's paired *t* test).

dependent Cl^- uptake into synaptoneurosomes by fairly low concentrations of EtOH in early studies (13) might have been due to the presence of extrasynaptic $\alpha_6\beta_3\delta$- or $\alpha4\beta_3\delta$-subunit-containing receptors in these preparations and that the failure to see EtOH effects at such low concentrations in many preparations and laboratories since that time may be explained by research focusing on recombinant receptors containing γ-subunits and *in vivo* studies on synaptic GABARs. Apart from real (with the α_4-subunit) or apparent difficulties (because GABA being only a partial agonist with low efficacy) to express δ-subunit-containing receptors in recombinant systems, δ-subunit-containing receptors may not have received attention because they make up only a fairly small fraction of GABARs in the mammalian brain, with each $\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$ estimated to contribute $<5\%$ to the total number of GABARs (34).

While this work was in progress, it was reported that $\alpha_4\beta_2\delta$-receptors expressed in *Xenopus* oocytes are enhanced by surprisingly low EtOH concentrations; threshold activation was reported to occur at 0.1 mM (11), a concentration 300 times lower than we observed with $\alpha_6\beta_2\delta$ or $\alpha4\beta_2\delta$ GABARs. These workers also showed a bell-shaped dose–response with the EtOH effect declining at 10 mM, and report (in their supporting data) that $\alpha_4\beta_3\delta$-receptors, which were the most sensitive to EtOH in our hands, did not give functional expression in oocytes. In addition, the GABA-EC_{20} for their $\alpha_4\beta_2\delta$-receptors is almost 10 times lower than in our experiments and reported in the literature (19, 27). It remains to be determined what accounts for these discrepancies. One possibility is the differences in the cDNAs used, particularly the functionality of their α_4-subunit.

Tonic inhibitory K^+ and Cl^- Currents Modulated by Anesthetics and Ethanol: Mutant Mice and Compensatory Mechanisms. Extrasynaptic GABARs are high-affinity receptors activated by ambient GABA concentrations (thought to be in the range of 0.5–1 μM), and their activation usually leads to a decrease in neuronal excitability. Functionally, extrasynaptic GABA receptors are similar to the "background K^+ channels," which comprise two main families: the two pore K^+ channels (TREK and TASK) and G protein-coupled inward rectifier K^+ (GIRK) channels (see Fig. 4). These K^+ channels are candidate targets for actions of volatile anesthetics (35, 36).

GABAR α_6-subunit knock-out mice not only lack α_6- but also δ-subunit protein in cerebellar granule cells (22), and lose "tonic" GABAR currents (37). Cerebellar granule cells compensate for this loss of extrasynaptic GABA receptors in $\alpha_6(-/-)$ mice by increasing their background leak conductance by expressing K^+ channels with properties characteristic for the two-pore-domain K^+ channel TASK1 (37). The up-regulation of TASK1 and possibly other proteins may explain why $\alpha_6(-/-)$ mice do not show motor deficits or changes in response to EtOH or other general anesthetics, including barbiturates (38). Although in β_3-subunit knock-out mice a significant reduction occurred in etomidate sensitivity of loss of righting reflex (39), the fairly moderate reduction is surprising given that mice carrying a single knock-in point mutation in the β_3-subunit (N256M, which makes β_3-containing receptors insensitive to etomidate *in vitro*) essentially eliminates etomidate- and propofol-induced anesthesia (40).

Consistent with the notion that δ GABARs are important targets for anesthetics, mice globally lacking the GABAR δ-subunit show not only a reduction in sensitivity to neurosteroid anesthetics but also a reduction in etomidate sensitivity (41). $\delta(-/-)$ mice also show defects in their behavioral responses to ethanol, with reduced ethanol consumption, attenuated withdrawal from chronic ethanol exposure, and reduced seizure-protective effects of ethanol (42). However, these mice still have normal anxiolytic and hypothermic EtOH responses and develop both chronic and acute tolerance. Taken together, compensatory homeostatic mechanisms may mask EtOH effects in straight α_6 and β_3 knock-out mice and some of the EtOH effects in δ-subunit knock-out animals. Based on our findings, it may be interesting to reevaluate EtOH and anesthetic effects in these knock-out animals.

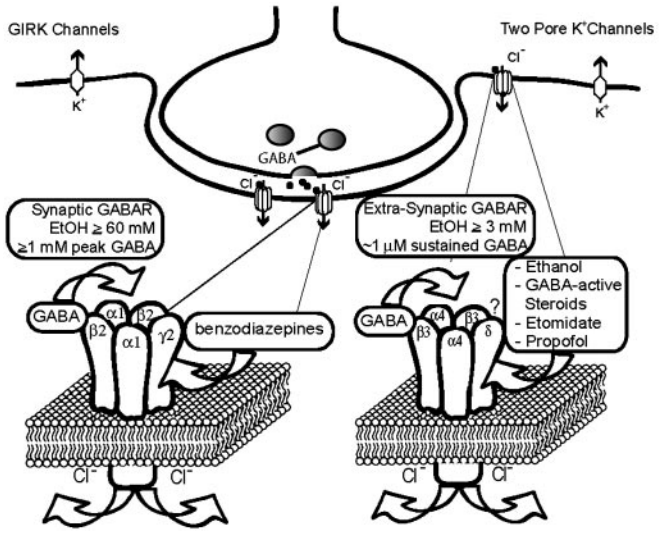

Fig. 4. Synaptic versus extrasynaptic receptors. Synaptic receptors (shown is the most prevalent $\alpha_1\beta_2\gamma_2$ synaptic GABARs) respond to saturating GABA (>1 mM peak GABA concentrations) and show high efficacy but fairly low potency (45). In contrast, extrasynaptic receptors (composed of $\alpha_4\delta$- or $\alpha_6\delta$- and most likely β_3-subunits) are activated by persistent and usually nonsaturating ambient GABA concentrations (0.5–1 μM), and, even at saturating GABA concentrations, are characterized by low-current levels (high-potency, low-efficacy receptors). We suggest a model where EtOH and other anesthetics lead to an increase in GABA efficacy (increase in open probability and/or possibly single-channel conductance), which leads to increased Cl^- current. A massive increase in GABA-activated Cl^- conductance by anesthetics could completely silence neurons expressing δ-subunit-containing GABARs, thereby producing anesthesia. The activation of extrasynaptic GABARs is functionally equivalent to activation of background K^+ channels. G protein-coupled inwardly rectifying K^+ (GIRK) channels have been shown to respond to fairly low concentrations of EtOH (3, 4) and may mediate EtOH analgesic actions (5). GIRK channels may also contribute to the anesthetic actions of volatile anesthetics (48, 49). The two-pore K^+ channels, TASK1 and TREK, are likely targets for volatile anesthetics (35, 36, 50, 51). The functional similarity between extrasynaptic GABARs and two-pore K^+ channels is supported by the finding that, in cerebellar granule cells, a background potassium channel (most likely TASK1), compensates for the loss of extrasynaptic GABARs in mice lacking the GABAR α_6-subunit (37).

We found that the β_3-subunit is required for high EtOH sensitivity of $\alpha_6\delta$- or $\alpha_4\delta$-subunit-containing GABARs and, therefore, if these receptors are important for *in vivo* EtOH effects, it would be expected that the β_3-subunit may be assembled in extrasynaptic $\alpha_6\beta\delta$ or $\alpha_4\beta\delta$ GABARs preferentially over other β-subtypes. The fact that the GABAR β_3(N256M) mutation in mice produces an almost complete loss of anesthetic etomidate's effects *in vi*vo and almost perfectly mirrors the loss of etomidate effect of this mutation in recombinant systems is surprising because β_2 (but not β_1)-subunit-containing receptors would be expected to be sensitive to etomidate *in vivo* as well (29, 40, 43). In marked contrast to the β_3(N256M) mice, the etomidate-insensitive β_2(N265S) mutant, when introduced into knock-in mice, does not abolish anesthesia, but eliminates most of the sedative effects of etomidate (30). An exclusive association of extrasynaptic $\alpha_4\delta$ (and $\alpha_6\delta$) with β_3-subunits (but not β_2- or β_1-subunits) to form the etomidate-sensitive anesthetic GABARs would provide a plausible explanation for the almost complete loss of anesthetic etomidate effects in the β_3(N256M)

mice. The depth and duration of sedation by etomidate and other GABAR-specific anesthetics is augmented by their actions on synaptic $\alpha\beta_2\gamma$-subunit-containing receptors (leading to increased IPSP/C decay times), and these effects are drastically reduced in the β_2(N265S) mutant mice (30). Along those lines, GABARs composed of $\alpha_1\beta_2\gamma_2$-subunits are thought to represent ≈50% of all GABARs in mammalian brain, and, because GABAR β_2- and β_3-subunits are each estimated to constitute ≈50% of total β-subunits (the β_1-subunit is a rare subunit) (34), most GABARs other than primarily synaptic $\alpha_1\beta_2\gamma_2$ GABARs must contain the β_3-subunit. These GABARs would include synaptic α_2- and α_3-containing receptors and probably most extrasynaptic receptors.

GABA as a Partial Agonist: Ethanol and Anesthetics Increase Efficacy. Oocytes expressing δ-subunit-containing receptors have only low-current levels. Although we cannot exclude that single-channel conductance increases with EtOH, etomidate, and THDOC (44), the 20-fold increase of GABA current produced by etomidate at saturating GABA concentrations is probably due to a low open probability with GABA alone. This low open probability provides an explanation for the apparent ''expression problems'' of δ-subunit-containing receptors. Therefore, GABA has only poor efficacy at δ-subunit-containing receptors and might be considered a partial agonist. The dramatic increase in GABA peak currents by anesthetics suggests that they may convert the partial agonist GABA into a full agonist at this receptor subtype.

Although GABAR-specific anesthetics (particularly etomidate) are more efficacious on δ-subunit-containing receptors than on γ-subunit-containing receptors (19), they also activate γ_2-containing (generally synaptic) GABARs [usually evaluated at nonsaturating (EC_{10} or EC_{50}) GABA concentrations]. The fact that inhibitory postsynaptic currents (and potentials) rarely show peak increases in response to GABAR-enhancing agents (benzodiazepine and anesthetics), but rather a slowed IPSP/C decay, suggests that (*i*) synaptic GABARs are usually activated by saturating (≈1 mM) GABA concentrations in the synaptic cleft and (*ii*) GABA is a full agonist at these receptors and leads to a nearly full receptor activation, which leaves only little or no room for peak current increases (26, 45–47). However, the stabilization of receptors in the open state by anesthetics may lead to the observed slowing of IPSP/C decay due to slower closing rates and/or decreased desensitization rates. It is therefore likely that the sedative benzodiazepine-like effects of GABAR-specific anesthetics are mediated by effects on synaptic, γ GABARs (see Fig. 4).

In summary, we show that GABARs composed of $\alpha_4\beta_3\delta$- and $\alpha_6\beta_3\delta$-subunits, which others have shown to be located extrasynaptically, are activated by low concentrations of EtOH, and that the β_3-subunit is required for effects of EtOH at these low concentrations. Previous studies apparently have not noted this potent action because δ-subunits were not studied in recombinant systems, and synaptic currents, generated by γ_2- rather than δ-containing GABARs, have been emphasized in neuronal studies. Because the same receptors that we find to be sensitive to low concentrations of EtOH also show dramatic enhancement with general anesthetics, we propose and argue for a simple model (Fig. 4), where extrasynaptic receptors are the primary targets for EtOH, other GABAR-specific anesthetics, and steroids. Stell *et al.*[‡] have shown that neurosteroids enhance tonic conductance generated by δ-containing GABARs. It will be important to determine whether EtOH (and other GABAR-specific anesthetics) enhance extrasynaptic currents at comparable concentrations in neurons expressing $\alpha_4\beta\delta$- and $\alpha_6\beta\delta$-receptors (but not in those neurons that do not) and if this action mediates physiological effects.

[‡]Stell, B. M., Brickley, S., Farrant, M., Tang, C. Y. & Mody, I. (2002) *Soc. Neurosci. Abstr.* 148.7.

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