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RESEARCH PAPER

$\alpha_1\beta_2\delta$, a silent $GABA_A$ receptor: recruitment by tracazolate and neurosteroids

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Background and purpose: This study investigated the $\alpha_1\beta_2\delta$ isoform of the $GABA_A$ receptor that is presumably expressed in the forebrain. The functional and pharmacological properties of this receptor combination are largely unknown.
Experimental approach: We expressed $\alpha_1\beta_2\delta$ $GABA_A$ receptors in *Xenopus laevis* oocytes. GABA-activated currents, in the presence and absence of modulators, were recorded using the two-electrode voltage clamp technique.
Key results: The $\alpha_1\beta_2\delta$ isoform of the $GABA_A$ receptor exhibited an extremely small GABA-mediated current. Tracazolate increased the current amplitude evoked by a half-maximal concentration (EC_{50}) of GABA by 59-fold. The maximum current was increased 23-fold in the presence of a saturating GABA concentration. Concomitant with the increase in the maximum, was a 4-fold decrease in the EC_{50}. Finally, a mutation in the second transmembrane domain of the δ subunit that increases receptor efficacy (L286S), eliminated the increase in the maximum GABA-activated current. The endogenous neurosteroid, tetrahydrodeoxycorticosterone (THDOC), also decreased the EC_{50} and increased the maximum current amplitude, although to a lesser degree than that of tracazolate.
Conclusions and implications: Taken all together, these findings indicate that the small GABA-mediated currents in the absence of the modulator are due to a low efficacy for activation. In the absence of modulators, $\alpha_1\beta_2\delta$ GABA receptors would be effectively silent and therefore contribute little to inhibition in the CNS. In the presence of tracazolate or endogenous neurosteroids however, this particular receptor isoform could exert a profound inhibitory influence on neuronal activity.
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Abbreviations: $GABA_A$ receptor, GABA type A receptor; THDOC, tetrahydrodeoxycorticosterone (3α,21-dihydroxy-5α-pregnan-20-one); Tracazolate, (4-(butylamino)-1-ethyl-6-methyl-1*H*-pyrazolo(3,4-β)pyridine-5-carboxylic acid ethyl ester)

Introduction

GABA is the major inhibitory neurotransmitter in the CNS. The GABA type A receptor ($GABA_A$ receptor) contains an integral chloride ion pore that can be gated (opened) by GABA and modulated by a variety of pharmacologically and clinically important drugs. $GABA_A$ receptors are composed of five subunits that can belong to several different subunit classes. So far, 19 different subunits have been identified in the mammalian brain; α_{1-6}, β_{1-4}, γ_{1-3}, δ, ε, θ, ρ_{1-3} (Barnard *et al.*, 1998). Depending on the subunit composition, these receptors exhibit distinct electrophysiological and pharmacological properties (Sieghart, 1995; Sanna *et al.*, 1997; Hevers and Luddens, 1998; Belelli *et al.*, 2002; Sieghart and Sperk, 2002; Yang *et al.*, 2005; Orser, 2006).

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The δ-subunit is one of the $GABA_A$ receptor subunits with a highly specific regional and subcellular distribution. It is abundant in cerebellar and dentate gyrus granule cells, some cortical areas, thalamic relay nuclei and the striatum (Benke *et al.*, 1991; Laurie *et al.*, 1992; Wisden *et al.*, 1992; Fritschy and Mohler, 1995; Sperk *et al.*, 1997; Pirker *et al.*, 2000; Peng *et al.*, 2002). GABA receptors containing $\alpha\beta\delta$-subunits have been localized to extra- and peri-synaptic membranes, exhibit a high sensitivity to GABA, show little desensitization and are believed to be one of the primary mediators of tonic inhibition. This tonic inhibition increases neuronal membrane conductance thereby modifying the spatial and temporal integration of excitatory signals (Brickley *et al.*, 2001; Macdonald *et al.*, 2004). It has been proposed that δ-subunits assemble with α_4-subunits in the forebrain and α_6-subunits in the cerebellum to form functional receptors. More recently, an association of δ with α_1 has been proposed and this particular combination may be a target for the actions of ethanol in the brain (Glykys *et al.*, 2007).

$GABA_A$ receptors containing αβδ subunits are modulated by many clinically important drugs, such as anxiolytics, anticonvulsants and sedative/hypnotics (Sundstrom-Poromaa *et al*., 2002; Wohlfarth *et al*., 2002; Wallner *et al*., 2003; Storustovu and Ebert, 2006; Winsky-Sommerer *et al*., 2007). Pyrazolopyridines (that is, tracazolate (4-(butylamino)-1-ethyl-6-methyl-1*H*-pyrazolo(3,4-β)pyridine-5-carboxylic acid ethyl ester), etazolate and cartazolate) are one such class of compounds that represent a chemically unique group of non-sedative anxiolytic agents (Barnes *et al*., 1983; Patel *et al*., 1985; Young *et al*., 1987; Thompson *et al*., 2002). Tracazolate demonstrates a wider separation between sedative and therapeutic doses than do benzodiazepines and appears to cause fewer adverse interactions than the benzodiazepines in combination with barbiturates and alcohol. Tracazolate can potentiate or inhibit recombinant $GABA_A$ receptor function depending on subunit combination. It has been shown that the nature of the third subunit (γ_{1-3}, δ or ε) within the receptor complex is critical in determining the functional response of tracazolate (Thompson *et al*., 2002).

Neurosteroids (including pregnanolone and allopregnanolone) are also powerful modulators of αβδ-containing $GABA_A$ receptors (Adkins *et al*., 2001; Brown *et al*., 2002; Wohlfarth *et al*., 2002). Tetrahydrodeoxycorticosterone (3α,21-dihydroxy-5α-pregnan-20-one) (THDOC), a metabolite of deoxycorticosterone (Crawley *et al*., 1986; Bitran *et al*., 1991; Kokate *et al*., 1994; Lambert *et al*., 1995; Reddy, 2003), increases the maximal GABA-evoked current several fold and converts αβδ $GABA_A$ receptor responses to highly desensitizing, slowly deactivating currents (Bianchi *et al*., 2002). This latter result would appear to be in agreement with a behavioural study using δ-knockout mice, which demonstrated that a global deletion of the δ-subunit of the $GABA_A$ receptor resulted in a decrease in the sensitivity of mice to the sedative/hypnotic, anxiolytic and proabsence effects of neuroactive steroids (Mihalek *et al*., 1999; Spigelman *et al*., 2002).

$\alpha_1\beta_2\delta$ receptors, although presumed to exist (Mody, 2004), have not been well investigated. This study was designed to investigate the functional characteristics of $\alpha_1\beta_2\delta$ $GABA_A$ receptors and the nature of its modulation by tracazolate and neurosteroids. Here we show that this $GABA_A$ receptor combination exhibits an extremely low efficacy for GABA-mediated activation. However, tracazolate and THDOC both increase the maximal current amplitude even in the presence of saturating GABA concentrations. We propose that these receptors, owing to their low efficacy, would be essentially silent, but upon exposure to neurosteroids or compounds, such as tracazolate, could exert a profound inhibition in the brain.

Materials and methods

cDNA cloning and in vitro *transcription*

Wild-type rat GABA receptor subunits α_1, β_2, γ_2 and δ were cloned into the pGEMHE vector (Liman *et al*., 1992). The cDNA for each construct was linearized by *Nhe*I digestion. The cDNAs were then transcribed and capped using standard *in vitro* transcription techniques (mMACHINE T7 kit; Ambion, Austin, TX, USA). cRNAs were purified and resuspended in diethyl pyrocarbonate-treated water.

Site-directed mutagenesis

The mutant δ L286S was made by the QuikChange method of site-directed mutagenesis using the KOD DNA Polymerase Kit (Novagen, Darmstadt, Germany). Successful mutagenesis was verified by sequencing.

Oocyte preparation and receptor expression

All procedures involving the *Xenopus* were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio. *Xenopus laevis* (*Xenopus* I, Ann Arbor, MI, USA) were anaesthetized by MS-222, and oocytes were surgically removed. The oocytes were placed in a solution consisting of 85.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 50 U ml^{-1} penicillin and 50 mg ml^{-1} streptomycin, pH 7.5. Oocytes were dispersed in this solution without Ca^{2+}, but in the presence of 0.25% Collagenase A (Roche Diagnostics, Indianapolis, IN, USA). After isolation, Stage VI oocytes were thoroughly rinsed and maintained at 16 °C in the above-mentioned solution with added Ca^{2+}. Micropipettes for injecting cRNA were pulled on a Sutter P87 horizontal puller and the tips were cut with forceps to 30–40 μm in diameter. To match the concentrations, cRNAs were electrophoresed each at several dilutions in a 1% agarose gel. Based on relative intensity of the bands, and in some cases measurement with a spectrophotometer, the cRNA concentrations were readjusted to be equal. cRNAs were injected into oocytes with a Nanoject microinjection system (Drummond Scientific) at a total volume of 50 nl. We injected oocytes with the following cRNA ratios: α_1:β_2:H_2O (1:1:8), α_1:β_2:γ_2 (1:1:8) and α_1:β_2:δ (1:1:8). The concentrations of cRNAs for α_1 and β_2 were 0.01–0.02 μg μl^{-1} and for δ cRNA, the concentration was 0.08–0.16 μg μl^{-1}. We injected an eightfold excess of δ-encoding or γ_2-encoding cRNAs to ensure that a majority of the expressed receptor complexes contained all three subunits.

Voltage-clamp experiments

Two-electrode voltage-clamp procedures were used for current recording 2 or 3 days after cRNA injection. The oocyte was placed in a small volume chamber with a continuous perfusion system, as described previously (Amin and Weiss, 1994). The flow rate in this chamber is $\approx$120 μl s^{-1}. The δ-containing receptors show very little desensitization, but the γ-containing receptors, at high agonist concentrations, desensitize rapidly. Given this rapid desensitization and the slow flow rate in the chamber, the peak of the GABA-activated currents will be attenuated. In all cases, current amplitudes were measured at the peak. The normal extracellular recording solution (OR2) contained 92.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.5, adjusted with NaOH. Recording microelectrodes were fabricated from thin-walled glass micropipettes (A-M Systems, Carlsborg, WA, USA) on a Sutter P-87 horizontal puller (Sutter, Novato, CA, USA) and filled with 3 M KCl (resistances were 1–3 MΩ). The oocytes were voltage clamped at −70 mV using a GeneClamp 500B (Axon Instruments, Foster City, CA, USA). On-line

digitization of the signal at 20 Hz was carried out with the ITC-16 data acquisition board (Instrutech, Long Island, NY, USA) and Igor software (Wavemetrics, Lake Oswego, OR, USA). We applied GABA for 15–20 s for $\alpha_1\beta_2$ $GABA_A$ receptors and for 20 s for $\alpha_1\beta_2\delta$ $GABA_A$ receptors. The duration of co-application of GABA with tracazolate or THDOC was 40 s, slightly longer than with GABA alone as the modulation had slower kinetics than the activation. In comparing the modulation of $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$ receptors, we used 3 μM GABA to activate the receptors. In earlier experiments, this GABA concentration was approximately the EC_{50} for both receptor subtypes. When all the data were pooled after the modulation studies, the EC_{50}s for the two combinations diverged, so that 3 μM GABA represented a retrospective fractional activation of 0.67 and 0.34 for $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$. This difference should be taken into account when comparing the fractional potentiation. However, our primary purpose for comparing the two receptor combinations was to confirm that the δ-subunit was indeed a functional component of the recombinant $\alpha_1\beta_2\delta$ receptors. Temperature-dependent experiments were performed using the heater/cooler apparatus N 640352, model CL-100 and N 640353, model SC-20 (Warner Instruments, Hamden, CT, USA).

Data analysis

The dose–response relationship of the GABA-induced current in recombinant $GABA_A$ receptors was least squares fit to the following equation:

$$\text{Activation} \quad I = I_{max}/(1 + (EC_{50}/[GABA])^{nH})$$

where the GABA-induced current (I) is a function of the GABA concentration, EC_{50} is the GABA concentration required for inducing a half-maximal current, *nH* is the Hill coefficient, and I_{max} is the maximum current. The maximum current was then used to normalize the dose–response curve for each individual oocyte. For activation, the average of the normalized currents for each GABA concentration was used to plot the data. All the data are presented as mean ± s.e.m. For the reported parameters such as EC_{50}s and Hill coefficients, the reported mean ± s.e.m. represents the averages of the individual fits.

Drugs and solutions

GABA (Calbiochem, San Diego, CA, USA) stock solution (100 mM) was prepared daily from the solid. Tracazolate and THDOC, both obtained from Sigma, were prepared in DMSO at 100 mM and frozen in aliquots. The working concentrations were freshly prepared from the stock solution immediately before use.

Results

Small currents in oocytes expressing $\alpha_1\beta_2\delta$ $GABA_A$ receptors

Xenopus laevis oocytes were injected with cRNA encoding for either $\alpha_1\beta_2$- or $\alpha_1\beta_2\delta$-subunits. We expressed $\alpha_1\beta_2$ receptors for the sake of comparison with $\alpha_1\beta_2\delta$ receptors in order to ensure that the δ-subunit was being expressed. After 1–3 days, the oocytes were voltage clamped and exposed to increasing concentrations of GABA to record GABA-mediated currents and assess the sensitivity for activation. Figure 1a shows representative currents induced by application of different GABA concentrations to $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$ receptor combinations. Although roughly equivalent amounts of cRNA were injected in the two cases, the maximum GABA-mediated current amplitude (I_{max}) for $\alpha_1\beta_2\delta$ was 50-fold less than that of $\alpha_1\beta_2$; 55 ± 5 nA ($n = 39$) versus 2740 ± 310 nA ($n = 23$; Figure 1c). In addition, $\alpha_1\beta_2\delta$ receptors exhibited a lower sensitivity to GABA, with the GABA concentration required for half-maximal activation (EC_{50}) being about twice that for $\alpha_1\beta_2$ receptors (Figure 1b; Table 1). Although we are unable to rule out the possibility of a sub-population of $\alpha_1\beta_2$ receptors along with $\alpha_1\beta_2\delta$, this difference in EC_{50} confirms that the δ-subunit was indeed being incorporated into the receptor. We also compared the maximal current amplitude of $\alpha_1\beta_2\delta$ with that of the prototypical $\alpha_1\beta_2\gamma_{2s}$ $GABA_A$ receptor combination. In this case, the maximum GABA-mediated current in response to a saturating concentration of GABA was 126-fold higher for $\alpha_1\beta_2\gamma_{2s}$ as compared to $\alpha_1\beta_2\delta$ (Figure 1c).

Tracazolate increases the maximum current amplitude of $\alpha_1\beta_2\delta$-containing $GABA_A$ receptors

It has previously been shown that tracazolate, a non-benzodiazepine anxiolytic, modulates GABA receptors containing the δ-subunit (Thompson *et al*., 2002). We therefore tested if tracazolate can modulate the GABA-mediated activation of $\alpha_1\beta_2\delta$ receptors. Oocytes expressing

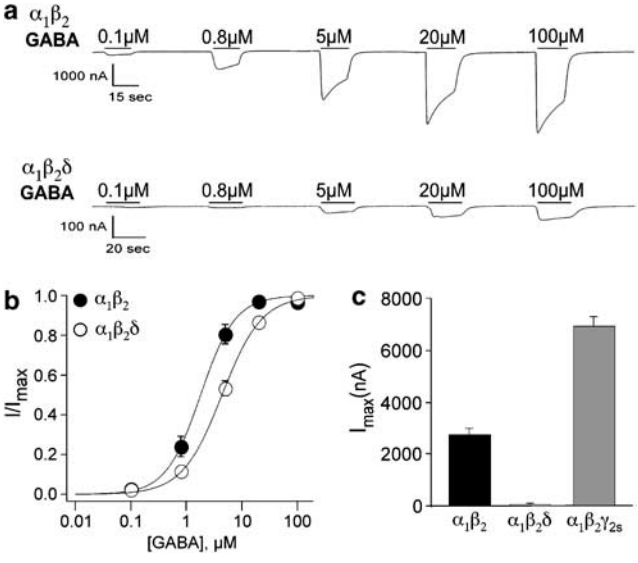

Figure 1 Representative recordings of currents in response to increasing concentrations of GABA on $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$ receptors. (**a**) GABA-mediated currents from oocytes expressing $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$ receptors are shown. (**b**) Concentration–response curves for GABA on oocytes expressing $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$ receptors. The continuous line is the best fit of the Hill equation yielding EC_{50}s of 1.8 ± 0.1 ($n = 16$) and 4.4 ± 0.1 ($n = 20$) for $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$, respectively. All currents were normalized to the maximal GABA-induced current in the same oocyte. (**c**) Bar graphs plotting the maximum currents of $\alpha_1\beta_2$ ($I_{max} = 2736 \pm 306$, $n = 23$) and $\alpha_1\beta_2\delta$ ($I_{max} = 55 \pm 5$, $n = 39$) $GABA_A$ receptors and $\alpha_1\beta_2\gamma_{2s}$ ($I_{max} = 6949 \pm 354$, $n = 42$). Although similar amounts of cRNA were injected, the currents were much larger in the $\alpha_1\beta_2$- and $\alpha_1\beta_2\gamma_{2s}$-expressing oocytes.

Table 1 Effects of tracazolate (3–20 μM) on EC_{50}s and Hill coefficients of $GABA_A$ receptor combinations

Combination	*EC_{50} (μM)*	*Hill*	n	*EC_{50} (μM) + 3 μMTrac*	*Hill*	n	*EC_{50} (μM) + 10 μMTrac*	*Hill*	n	*EC_{50} (μM) + 20 μMTrac*	*Hill*	n
$\alpha_1\beta_2$	1.8 ± 0.1	1.4 ± 0.1	16	0.4 ± 0.1*	1.1 ± 0.7	11	0.2 ± 0.1*	0.6 ± 0.2	10	0.1 ± 0.0*	1.3 ± 0.3	6
$\alpha_1\beta_2\delta$	4.4 ± 0.1	1.2 ± 0.0	20	2.8 ± 0.6*	1.0 ± 0.1	11	1.0 ± 0.3*	0.9 ± 0.2	10	0.4 ± 0.1*	1.8 ± 0.3	6
$\alpha_1\beta_2\delta$(9′L/S)	2.8 ± 0.1	1.3 ± 0.1	20	ND	ND	ND	0.4 ± 0.0	1.4 ± 0.2	10	ND	ND	ND

ND, not determined. *Significantly different from values without tracazolate; $P<0.02$.

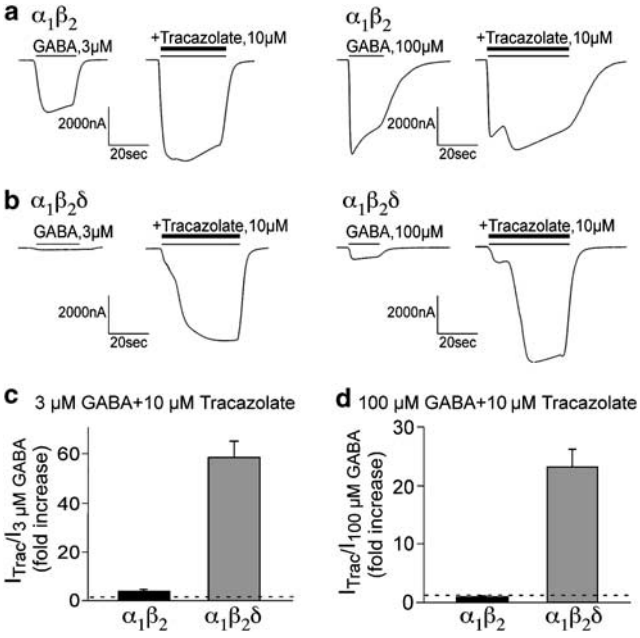

Figure 2 The action of tracazolate on the peak current amplitude from oocytes expressing $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$ $GABA_A$ receptors. (**a**) $\alpha_1\beta_2$ receptor currents at 3 μM (left) and 100 μM GABA (right) in the absence and presence of 10 μM tracazolate. (**b**) $\alpha_1\beta_2\delta$ receptor currents at 3 μM (left) and 100 μM GABA (right) in the absence and presence of 10 μM tracazolate. (**c**) The fold increase by tracazolate is shown for 3 μM GABA for $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$. Although a modest 3.9 ± 0.8-fold ($n=13$) increase was observed for $\alpha_1\beta_2$, the $\alpha_1\beta_2\delta$ currents were increased by 58.7 ± 6.3-fold ($n=14$). (**d**) The fold increase by tracazolate is shown with a saturating concentration of GABA (100 μM) for $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$. $\alpha_1\beta_2$ receptors show no potentiation (1.0 ± 0.1-fold, $n=54$), whereas the $\alpha_1\beta_2\delta$ currents were increased by 23.4 ± 3.0-fold ($n=54$).

$\alpha_1\beta_2$- and $\alpha_1\beta_2\delta$-subunits were tested with 3 and 100 μM (saturating) GABA without and with the co-application of tracazolate (10 μM). Neither receptor combination was directly activated by 10 μM tracazolate alone. The GABA-gated currents for $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$ are shown in Figures 2a and b, respectively. The fold increases in the peak current amplitudes imparted by tracazolate are plotted in Figures 2c and d. Note the inflection in the currents when GABA and tracazolate are applied simultaneously (obvious in subsequent figures as well). The likely interpretation for this inflection is differences in the activation/modulation rates for GABA and tracazolate. More specifically, the activation by GABA precedes in time the modulation by tracazolate. This is supported by the observation that the inflection is not present when we preincubate oocytes with tracazolate and then apply GABA plus tracazolate (data not shown). For $\alpha_1\beta_2$ receptors, tracazolate increased the peak current amplitude in response to 3 μM GABA by 3.9 ± 0.8-fold ($n=13$) (Figure 2c). The current in response to 100 μM GABA was not potentiated by tracazolate ($n=54$; Figure 2d). In contrast to these observations for $\alpha_1\beta_2$, we observed a profound potentiation of the GABA-mediated current amplitude by tracazolate at both the lower and higher GABA concentrations for the $\alpha_1\beta_2\delta$ receptor. Tracazolate increased the peak current amplitude in response to 3 or 100 μM GABA by almost 60-fold ($n=14$, $P<0.001$; Figure 2c) and about 20-fold ($n=54$, $P<0.00001$; Figure 2d), respectively. Despite the low level of GABA-mediated activation described in Figure 1, $\alpha_1\beta_2\delta$ receptors are indeed highly expressed on the cell surface. These findings suggest that $\alpha_1\beta_2\delta$ receptors are of extremely low efficacy for GABA-mediated activation (that is, GABA behaves as a weak partial agonist). We will provide additional support for this in a later section.

Tracazolate enhances the sensitivity of $\alpha_1\beta_2\delta$-containing $GABA_A$ receptors to GABA

To probe further into the mechanism of action of tracazolate, GABA concentration–response curves were constructed in the absence and presence of 10 μM tracazolate on oocytes expressing $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$ $GABA_A$ receptors (Figures 3a and b, respectively). $\alpha_1\beta_2$-Containing receptors were ninefold more sensitive to GABA in the presence of tracazolate (Figure 3a; EC_{50} values in Table 1). $\alpha_1\beta_2\delta$-Containing receptors were fourfold more sensitive to GABA in the presence of 10 μM tracazolate (Figure 3b; Table 1). The insets in Figures 3a and b show normalized $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$ dose–response curves so the increase in GABA sensitivity is more easily visualized. We obtained qualitatively similar results for both receptor combinations in the presence of 3 and 20 μM tracazolate (Table 1). Thus, although both receptor combinations demonstrate an increase in sensitivity to GABA with tracazolate, the profound increase in the maximum requires the presence of the δ-subunit.

THDOC potentiates GABA receptors comprised of $\alpha_1\beta_2\delta$-subunits

It is known that neurosteroids potentiate GABA receptors containing the δ-subunit (Brown *et al.*, 2002; Wohlfarth *et al.*, 2002; Bianchi and Macdonald, 2003). Oocytes expressing $\alpha_1\beta_2$ or $\alpha_1\beta_2\delta$ $GABA_A$ receptors were tested with 1 and 100 μM GABA without and with 1 μM THDOC. Neither receptor combination was directly activated by 1 μM THDOC alone. THDOC potentiated both $\alpha_1\beta_2$ (Figure 4a) and $\alpha_1\beta_2\delta$

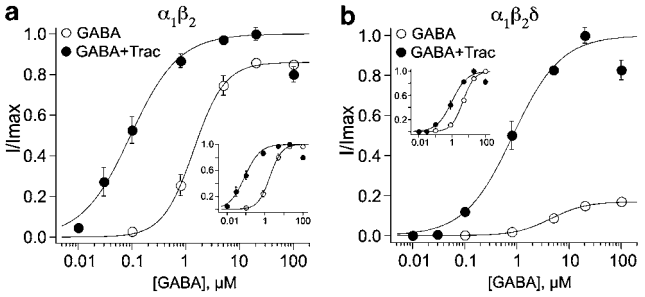

Figure 3 Tracazolate increases the sensitivity of $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$ $GABA_A$ receptors. (**a**) Concentration–response curves for GABA were obtained in the absence and presence of 10 μM tracazolate for oocytes expressing $\alpha_1\beta_2$ receptors. Very little change in the maximum current was observed, although 10 μM tracazolate decreased the EC_{50} from 1.8 ± 0.1 μM ($n=16$) to 0.2 ± 0.1 μM ($n=10$). The inset shows the normalized dose–response curves in order to facilitate comparison of the EC_{50}s. (**b**) Concentration–response curves for GABA were obtained in the absence and presence of 10 μM tracazolate for oocytes expressing $\alpha_1\beta_2\delta$ receptors. The increase in the maximum and the decrease in EC_{50} in the presence of 10 μM tracazolate (from 4.4 ± 0.1 μM, $n=20$ to 1.0 ± 0.1 μM, $n=10$) is obvious when the dose–response relationships were normalized and replotted (inset). The error bars for the open circles in (**b**) are smaller than the symbols.

(Figure 4b) currents elicited by low (1 μM) GABA concentrations. However, as evident in Figure 4c, the mean enhancement was significantly larger for $\alpha_1\beta_2\delta$ $GABA_A$ receptors than for $\alpha_1\beta_2$ receptors ($P<0.0005$). With a saturating concentration of GABA (100μM), THDOC enhanced $\alpha_1\beta_2\delta$ currents 3.4 ± 0.2-fold ($n=12$), whereas $\alpha_1\beta_2$ currents exhibited less enhancement (Figure 4d; $n=16$ $P<0.05$). For $\alpha_1\beta_2\delta$ receptors, THDOC modulation produced substantially larger currents than the maximal currents produced by GABA alone.

To further investigate the actions of THDOC, dose–response curves for GABA were constructed in the absence and presence of 1 μM THDOC on oocytes expressing $\alpha_1\beta_2$ or $\alpha_1\beta_2\delta$ $GABA_A$ receptors (Figures 5a and b, respectively). THDOC produced a leftward shift in the GABA dose–response curves for both $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$ receptors. As seen in Figure 5a, $\alpha_1\beta_2$-containing receptors were sixfold more sensitive to GABA in the presence of THDOC ($EC_{50}=0.3 \pm 0.1$ μM, $n=4$) than without THDOC ($EC_{50}=1.8 \pm 0.1$ μM, $n=16$; $P<0.00005$). $\alpha_1\beta_2\delta$-Containing receptors were 2.6-fold more sensitive to GABA in the presence of THDOC with EC_{50}s of 4.4 ± 0.1 μM ($n=20$) and 1.7 ± 0.2 μM ($n=5$; $P<0.005$) without and with THDOC, respectively.

Low efficacy was not due to the temperature dependence of $\alpha_1\beta_2\delta$-containing $GABA_A$ receptors

We considered the possibility that the low efficacy of $\alpha_1\beta_2\delta$ GABA receptors might be due to the lower recording temperature (≈24 °C). Perhaps at physiological temperatures where these receptors normally exist, the efficacy is higher. For example, if the opening rate were highly temperature dependent and increased with increasing temperature, this could account for the observed small currents and proposed low efficacy. To test this possibility, we recorded

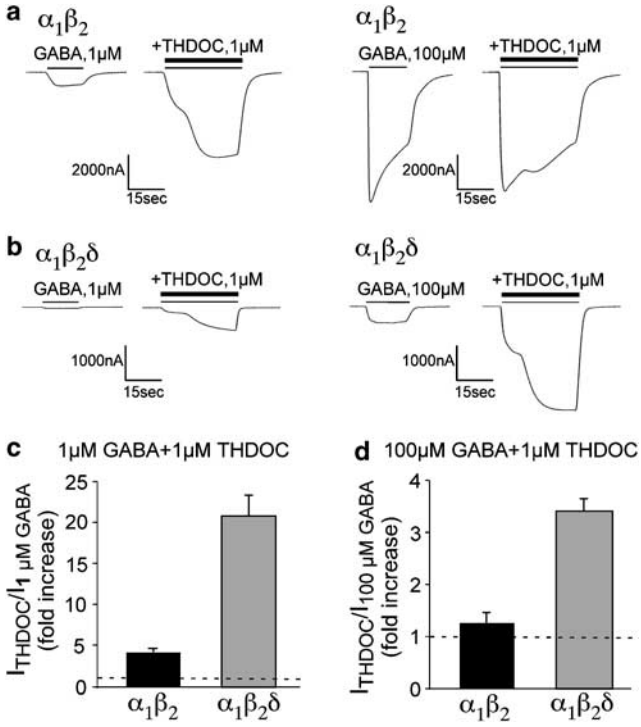

Figure 4 The actions of THDOC (1 μM) on the peak current amplitude for oocytes expressing $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$ $GABA_A$ receptors. (**a**) $\alpha_1\beta_2$ receptor currents at 1 μM (left) and 100 μM GABA (right) in the absence and presence of 1 μM THDOC. (**b**) $\alpha_1\beta_2\delta$ receptor currents at 1 μM (left) and 100 μM GABA (right) in the absence and presence of 1 μM THDOC. (**c**) The fold increase by THDOC is shown for 1 μM GABA for $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$. $\alpha_1\beta_2$ receptor currents were increased fourfold in the presence of 1 μM GABA in comparison to the 20-fold increase for $\alpha_1\beta_2\delta$. (**d**) The fold increase by THDOC is shown for a saturating concentration of GABA (100 μM) for $\alpha_1\beta_2$ and $\alpha_1\beta_2\delta$. $\alpha_1\beta_2$ exhibited a modest 1.25-fold increase ($n=16$), whereas the $\alpha_1\beta_2\delta$ currents were increased by 3.4 ± 0.2-fold ($n=15$).

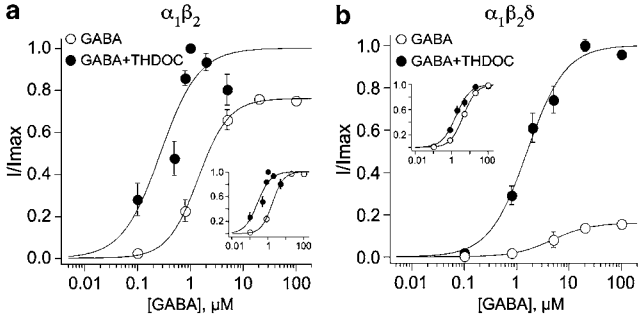

Figure 5 THDOC increased the sensitivity of the $GABA_A$ receptors. (**a**) Concentration–response curves for GABA were obtained in the absence and presence of 1 μM THDOC for oocytes expressing $\alpha_1\beta_2$ receptors. THDOC decreased the EC_{50} from 1.8 ± 0.1 μM ($n=16$) to 0.3 ± 0.1 μM ($n=4$). The inset shows the data normalized to facilitate the comparison of the shift in sensitivity. (**b**) Concentration–response curves for GABA were obtained in the absence (open circles) and presence (filled circles) of 1 μM THDOC for oocytes expressing $\alpha_1\beta_2\delta$ receptors. The profound increase in the maximum amplitude was observed, and the decrease in EC_{50} imparted by 1 μM THDOC (from 4.4 ± 0.1 μM, $n=20$ to 1.7 ± 0.2 μM, $n=5$) was obvious when the dose–response relationships were normalized and replotted (inset).

GABA-evoked currents in oocytes expressing $\alpha_1\beta_2\delta$ receptors across a range of temperatures. For these experiments, we used saturating concentrations of GABA (100 μM). As shown in Figure 6 (a plot of the maximum current as a function of recording temperature), we actually observed a slight decrease in the maximum current amplitude at higher temperatures. In these acute experiments, it is unlikely that temperature was affecting the translation or surface expression of the receptor. A more likely explanation is that this decrease in current amplitude is due to an increase in receptor desensitization. Nevertheless, the low efficacy seems to be an inherent property of this receptor combination and not a consequence of the recording temperature.

Additional evidence for low efficacy

We previously employed the mutation of a conserved leucine in the second transmembrane domain to determine the stoichiometry of the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor (Chang and Weiss, 1999). Subsequent studies demonstrated that the increase in sensitivity to GABA imparted by this mutation was due to an enhanced gating or efficacy (Chang *et al.*, 1996; Bianchi and Macdonald, 2001). Indeed, when we introduced this mutation into the δ subunit (L286S), we noted a profound increase in the amplitude of GABA-mediated currents (L286S; 4547 ± 184 nA; $n = 39$) compared with the wild-type $\alpha_1\beta_2\delta$ receptor (Figure 1c; 55 ± 5 nA; $n = 39$), suggesting that this mutation increased receptor efficacy. Figure 7 shows the GABA dose–response relationships before and after the addition of 10 μM tracazolate. Although the increase in sensitivity imparted by tracazolate was still present (Table 1), the maximum currents were hardly affected (1.4 ± 0.0-fold increase for L286S versus 1.0 ± 0.1-fold increase for wild type).

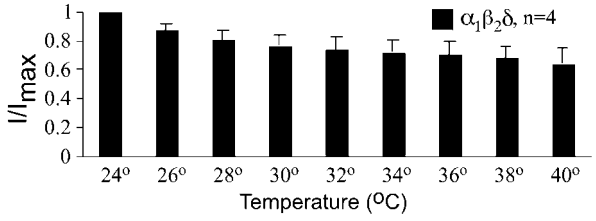

Figure 6 Low efficacy of $\alpha_1\beta_2\delta$ receptors is not due to the low recording temperatures. Maximal currents (100 μM GABA) were recorded from oocytes expressing $\alpha_1\beta_2\delta$ receptors and each of four oocytes was recorded across a range of temperatures. There was a slight decrease in the maximal current as temperature was increased, opposite to what would be expected if the low efficacy were due to the low recording temperatures.

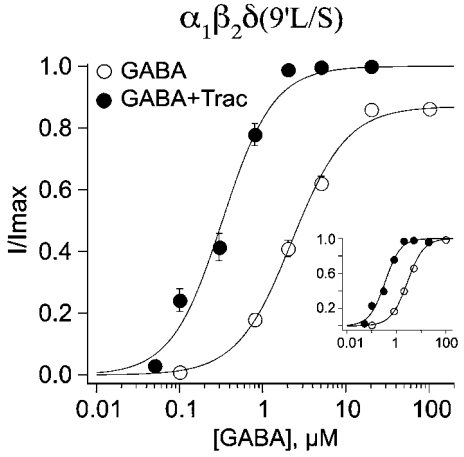

Figure 7 The 9′ leucine mutation (L286S) in the δ-subunit occludes the tracazolate-mediated increase in maximal amplitude. The mutant δ-subunit was co-expressed in oocytes with wild-type α_1 and β_2. Dose–response relationships were constructed in the absence and presence of 10 μM tracazolate. Although the leftward shift was still observed, the dramatic increase in the maximum current was absent. The EC_{50}s were 2.8 ± 0.1 ($n = 20$) and 0.4 ± 0.0 ($n = 10$) in the absence and presence of tracazolate, respectively.

Discussion and conclusions

$\alpha_1\beta_2\delta$ as a native receptor combination

Assembly of different combinations of GABA receptor subunits in different brain regions contributes to the diversity of GABA-mediated inhibition in the CNS (Macdonald and Olsen, 1994). The specific functional properties of δ-subunit-containing $GABA_A$ receptors, such as high sensitivity to GABA and low desensitization, allow them to be activated by the micromolar levels of GABA constantly present in the extracellular space (Lerma *et al.*, 1986; Saxena and Macdonald, 1996; Stell *et al.*, 2003; Mtchedlishvili and Kapur, 2006). In this regard, they can exert a tonic inhibitory influence. δ-Containing isoforms of $GABA_A$ receptors also play a particularly important role in the regulation of inhibition in the CNS by endogenous neurosteroids as well as behavioral responses to certain sedative/hypnotic and anxiolytic drugs (Mihalek *et al.*, 1999; Peng *et al.*, 2002; Wohlfarth *et al.*, 2002; Bianchi and Macdonald, 2003; Wallner *et al.*, 2003; Dibbens *et al.*, 2004).

The δ-subunit has been assumed to be predominately co-expressed with α_4- and/or α_6-subunits, whereas the α_1-subunit has been assumed to be co-expressed with γ_2. There have been reports, however, demonstrating that α_1, β_2 and δ subunits may be co-expressed in the same neurons (Mertens *et al.*, 1993; Pirker *et al.*, 2000; Mangan *et al.*, 2005). One study, using quantitative immunoblotting, has suggested the presence of an $\alpha_1\alpha_6\beta_2\delta$ receptor combination in rat cerebellum (Thompson *et al.*, 1996). An α_6 knockout, however, found no evidence for the existence of $\alpha_1\beta_x\delta$ receptors (Jones *et al.*, 1997). More recently, α_4 knockout studies have shown that α_1- and δ-subunits are co-expressed in hippocampal interneurons and may form functional receptors with the β_2-subunit. It was suggested that this receptor is a target for low concentrations of ethanol (Glykys *et al.*, 2007). Furthermore, changes in the expression of the δ-subunit, such as with progesterone exposure, could regulate the levels of these δ-containing receptors at opportune times (Mostallino *et al.*, 2006).

In terms of methodology, the most accepted approach to detect native subunit combinations is immunoprecipitation. Although this method may be appropriate for abundant populations of receptors, it may not be sensitive enough to discriminate minor receptor subtypes. Nevertheless, even if

the $\alpha_1\beta_2\delta$ combination is present as a minor $GABA_A$ receptor subtype in the adult CNS (McKernan and Whiting, 1996), given the strong potentiation reported here, this receptor could play an important role in the modulation of inhibition in the CNS via neurosteroids and modulators, such as tracazolate.

We compared the function and modulation of $\alpha_1\beta_2\delta$ receptors to that of $\alpha_1\beta_2$, primarily to demonstrate that the δ-subunit was indeed incorporated into the functional receptor. There is evidence, however, that $\alpha_1\beta_2$ may be expressed in hippocampal pyramidal neurons and account for up to 10% of the total extrasynaptic receptor pool (Mortensen and Smart, 2006). If so, the differential modulation of this combination by tracazolate and THDOC should be considered when evaluating the physiological actions of these modulators.

$\alpha_1\beta_2\delta$ is a low-efficacy receptor

The $\alpha_1\beta_2\delta$ receptor combination, when exogenously expressed, demonstrated extremely small GABA-activated currents. These currents were some 50-fold less than that of $\alpha_1\beta_2$ and 126-fold less than that of $\alpha_1\beta_2\gamma_{2s}$ with comparable amounts of injected cRNA. However, both tracazolate and THDOC produced a dramatic increase in the current amplitude for $\alpha_1\beta_2\delta$ receptors, even at saturating GABA concentrations. Furthermore, the action of these compounds on receptor sensitivity (EC_{50}) was minimal. In its simplest interpretation, these observations imply that the modulators are not altering receptor affinity, but rather modulating receptor gating. Furthermore, a mutation (L286S) in the δ-subunit that increased the efficacy of GABA and restored the current amplitude to levels comparable to that of $\alpha_1\beta_2\gamma_{2s}$ prevented the increase in amplitude mediated by tracazolate. Taken together, these findings indicate that the $\alpha_1\beta_2\delta$ receptor combination is likely to be a low-efficacy receptor. Other δ-containing receptors have been deemed low efficacy, such as $\alpha_1\beta_1\delta$ (Thompson *et al*., 2002) and $\alpha_1\beta_3\delta$ (Wohlfarth *et al*., 2002; Feng and Macdonald, 2004a, b). Thus, it appears that for αβδ isoforms in general, GABA behaves as a partial agonist (Adkins *et al*., 2001; Brown *et al*., 2002). The most detailed single-channel analysis to date on δ-containing recombinant GABA receptors ($\alpha_1\beta_3\delta$) indicated a low open channel probability and mean open time, as well as one less open state than that of $\alpha_1\beta_3\gamma_{2L}$ (Fisher and Macdonald, 1997; Akk *et al*., 2004a). We also observed small currents in response to GABA and profound modulation by tracazolate for $\alpha_1\beta_2\delta$ receptors and no tracazolate-mediated potentiation of $\alpha_1\beta_2\gamma_{2s}$ receptors when these two combinations were expressed in HEK293 cells (unpublished observations). Thus, the low efficacy of $\alpha_1\beta_2\delta$ receptors is not a consequence of the oocyte expression system.

Even at saturating concentrations of GABA, owing to this low efficacy, the $\alpha_1\beta_2\delta$ receptor would be essentially silent. Upon exposure to THDOC or tracazolate, however, this receptor combination could exert a profound inhibitory influence on excitability in the CNS. This provides a unique mechanism for recruiting inhibition without the cost of receptor trafficking to the surface or the activation of intracellular signaling cascades. Although we observed an increase in current amplitude by tracazolate and THDOC for $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$, this was only observed at low GABA concentrations and was due to a leftward displacement in the dose–response relationship. At saturating GABA concentrations, there was no increase in the maximum amplitude and a similar result has been obtained for $\alpha_1\beta_3\gamma_2$ receptors (Thompson *et al*., 2002). Given that GABA concentrations in the synaptic cleft are likely to far exceed saturating levels (Wardell *et al*., 2006), this provides a mechanism by which these modulators can selectively target δ-containing, extrasynaptic, tonic receptors.

Mechanism of modulation by tracazolate and THDOC

In the present study, 10 μM tracazolate imparted a 59-fold increase in the GABA-mediated current amplitude at low GABA concentrations (3 μM) and a 23-fold increase in the current amplitude at saturating GABA concentrations (100 μM). Other δ-containing receptors exhibit a strong potentiation to tracazolate, although less than that observed here for $\alpha_1\beta_2\delta$. For $\alpha_2\beta_3\delta$ (Yang *et al*., 2005) and $\alpha_1\beta_1\delta$ (Thompson *et al*., 2002) receptor combinations, tracazolate potentiated the maximum current approximately threefold.

Thompson *et al*. (2002) determined that the particular isoform of the β subunit is important for the actions of tracazolate on receptors. More specifically, tracazolate exhibits a higher sensitivity on $\alpha_1\beta_3\gamma_2$ receptors in comparison with $\alpha_1\beta_1\gamma_2$. Interestingly, whether tracazolate potentiates or inhibits the receptor depends upon the nature of the third subunit (γ_{1-3}, δ or ε). The presence of a γ_2 or δ subunit imparts a potentiation by tracazolate, the presence of an ε-subunit imparts an inhibition, and the presence of γ_1 or γ_2 can impart an intermediate profile with potentiation or inhibition depending on the concentrations of agonist and modulator. This is similar to what we observed here for the $\alpha_1\beta_2$ combination in the absence of a third subunit. Given our data and that of Thompson *et al*., it is clear that for the $\alpha_1\beta_x\delta$ combination, the sensitivity of modulation by tracazolate seems to be dependent on the β subunit present.

Thompson *et al*. (2002) have observed that under conditions where the receptor rapidly entered the desensitized state (for example, $\alpha_1\beta_{1-3}\varepsilon$ or high concentrations of GABA on $\alpha_1\beta_3\gamma_{2s}$), the functional response to tracazolate was inhibition, whereas in conditions with little desensitization (for example, $\alpha_1\beta_1\delta$ or low GABA concentration on $\alpha_1\beta_3\gamma_{2s}$) the functional response was potentiation. They propose that tracazolate displays higher affinity for the desensitized state than for the agonist bound state of the receptor. This mirrors our findings where we observed potentiation in $\alpha_1\beta_2\delta$, a combination that displays modest desensitization, and inhibition in $\alpha_1\beta_2\gamma_2$ at high agonist concentrations where a strong desensitization was evident. Although the hypothesis is certainly tempting, the inability to separate activation from desensitization makes a causal relationship difficult to confirm.

We also compared the actions of the endogenous neuroactive steroid THDOC on GABA receptors comprised of $\alpha_1\beta_2$- and $\alpha_1\beta_2\delta$-subunits. As was true for tracazolate, we observed a greater enhancement for the ternary complex, with increases in current amplitude of 20.7- and 3.4-fold for

$\alpha_1\beta_2\delta$ at an EC_{50} and saturating concentration of GABA, respectively.

The list of neuroactive steroids is long, and therefore, reconciliation between studies employing the various compounds on different GABA receptor subunit combinations can be difficult. What is clear is that there is strong evidence that neurosteroids have multiple sites of action on the GABA receptor (Akk *et al*., 2001; Morris and Amin, 2004; Ueno *et al*., 2004; Akk *et al*., 2004b; Wardell *et al*., 2006; Hosie *et al*., 2007). Recently, two domains have been identified and suggested to represent neurosteroid binding sites (Hosie *et al*., 2006). One binding domain is within the α-subunit, whereas the second binding domain is at the α-β-subunit interface. These two domains are presumed to represent sites for modulation and activation, respectively. Because the neurosteroids are hydrophobic, the neurosteroid binding site(s) is likely to include a region of the $GABA_A$ receptor polypeptide embedded within the membrane, although access to the site appears to be extracellular, as intracellular application of the neurosteroid is ineffective (Twyman and Macdonald, 1992). More recent evidence from patch clamp studies in which the neurosteroids were applied through various compartments, as well as imaging studies with a fluorescent neurosteroid analogue, indicates interaction with sites embedded in the membrane that can be accessed by lateral membrane diffusion (Akk *et al*., 2004b). Recent imaging studies indicate, however, that GABA-active steroids can accumulate both in the plasma membrane and the intracellular space (Akk *et al*., 2005).

Although single-channel kinetic analyses have been carried out on GABA receptors to gain further insight into the modulatory mechanism, a definitive model is still lacking (Akk *et al*., 2004b). Our macroscopic data agree with the studies of Bianchi and Macdonald (2003) that conclude a shift from low to high efficacy, similar to that assumed for tracazolate discussed earlier. In its simplest form, a low-efficacy receptor can be modelled by a kinetic scheme with a slow channel-opening rate. In this scheme, the resulting low open-channel probability (P_{open}) would underlie the small macroscopic GABA-mediated maximum currents. Although a quantitative validation of this mechanism would require a detailed single-channel analysis to derive the relevant rate constants, an increase in the opening rate by tracazolate could impart the observed profound increase in the maximum current, with a modest decrease in the EC_{50} (Figure 3b of Amin and Weiss, 1993).

Although not specifically examined in the present study, neurosteroids have been shown to directly activate the receptor, albeit with low efficacy compared to GABA. This adds neurosteroids to the growing list of GABA receptor modulators that both activate and potentiate the receptor (Rusch *et al*., 2004; Campo-Soria *et al*., 2006). As for other modulators (diazepam, flurazepam, zolpidem and etomidate), perhaps an allosteric model can serve to unify the activation and modulation processes.

In summary, the $\alpha_1\beta_2\delta$ GABA receptor subunit combination can be strongly modulated by the anxiolytic drug tracazolate and the endogenous neurosteroid THDOC. As this receptor combination is likely to be expressed in the forebrain (Dibbens *et al*., 2004) and cerebellum (Poltl *et al*., 2003), our findings suggest a major contribution of these receptors to the control of normal tonic inhibition and a potential for its therapeutic regulation.

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Conflict of interest

The authors state no conflict of interest.

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