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Enhanced Neurosteroid Potentiation of Ternary $GABA_A$ Receptors Containing the δ Subunit

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Attenuated behavioral sensitivity to neurosteroids has been reported for mice deficient in the $GABA_A$ receptor δ subunit. We therefore investigated potential subunit-specific neurosteroid pharmacology of the following $GABA_A$ receptor isoforms in a transient expression system: $\alpha1\beta3\gamma2L$, $\alpha1\beta3\delta$, $\alpha6\beta3\gamma2L$, and $\alpha6\beta3\delta$. Potentiation of submaximal $GABA_A$ receptor currents by the neurosteroid tetrahydrodeoxycorticosterone (THDOC) was greatest for the $\alpha1\beta3\delta$ isoform. Whole-cell GABA concentration–response curves performed with and without low concentrations (30 nM) of THDOC revealed enhanced peak $GABA_A$ receptor currents for isoforms tested without affecting the GABA EC_{50}. $\alpha1\beta3\delta$ currents were enhanced the most (>150%), whereas the other isoform currents were enhanced 15–50%. At a higher concentration (1 μM), THDOC decreased peak $\alpha1\beta3\gamma2L$ receptor current amplitude evoked by GABA (1 mM) concentration jumps and prolonged deactivation but had little effect on the rate or extent of apparent desensitization. Thus the polarity of THDOC modulation depended on GABA concentration for $\alpha1\beta3\gamma2L$ $GABA_A$ receptors. However, the same protocol applied to $\alpha1\beta3\delta$ receptors resulted in peak current enhancement by THDOC of >800% and prolonged deactivation. Interestingly, THDOC induced pronounced desensitization in the minimally desensitizing $\alpha1\beta3\delta$ receptors. Single channel recordings obtained from $\alpha1\beta3\delta$ receptors indicated that THDOC increased the channel opening duration, including the introduction of an additional longer duration open state. Our results suggest that the $GABA_A$ receptor δ subunit confers increased sensitivity to neurosteroid modulation and that the intrinsic gating and desensitization kinetics of $\alpha1\beta3\delta$ $GABA_A$ receptors are altered by THDOC.

Key words: $GABA_A$ receptor; δ subunit; neurosteroid; desensitization; single channel; gating

Fast synaptic inhibition in the mammalian CNS is mediated mainly by activation of $GABA_A$ receptor channels (Macdonald and Olsen, 1994; Whiting et al., 1995). $GABA_A$ receptor function is modulated by various clinically important drugs that act on allosteric modulatory sites (Macdonald and Olsen, 1994; Sieghart, 1995). For example, neurosteroids, which represent a class of molecules that are synthesized in the nervous system, have been demonstrated to have anxiolytic, hypnotic, anesthetic, and anticonvulsant effects (Baulieu and Robel, 1990; Paul and Purdy, 1992; Macdonald and Olsen, 1994; Lambert et al., 1995) and may be involved in memory enhancement, behavioral actions, and neuroprotection (Frye, 1995; Green et al., 2000; Yoo et al., 1996).

Several studies have shown that neurosteroids bind to $GABA_A$ receptors at sites different from GABA, benzodiazepines, and barbiturates (Gee et al., 1988; Turner et al., 1989) and can act as positive or negative modulators of receptor function (Majewska et al., 1986; Gee et al., 1988; Puia et al., 1990; Gee and Lan, 1991; Park-Chung et al., 1999). Neurosteroid enhancement of submaximal $GABA_A$ receptor currents occurs through increases in both channel open frequency and open duration (Puia et al., 1990; Twyman and Macdonald, 1992). At high concentrations, neurosteroids can directly activate $GABA_A$ receptor channels (Lambert et al., 1995).

The $GABA_A$ receptor is a pentameric structure formed by the coassembly of subunit polypeptides from a large multigene family (McKernan and Whiting, 1996; Barnard et al., 1998) that are differentially expressed both temporally and spatially throughout the brain (Zheng et al., 1993, 1995). This heterogeneous expression confers specific physiological and pharmacological properties of $GABA_A$ receptors (Sigel et al., 1990; Mathews et al., 1994). For example, it has been demonstrated that the presence of α and γ subunits can affect neurosteroid modulation. The α subunit subtype was found to influence efficacy, whereas the γ subunit subtype influenced both efficacy and EC_{50} for neurosteroid interaction with $GABA_A$ receptors (Gee and Lan, 1991; Lan et al., 1991; Sapp et al., 1992). Also, Zhu et al. (1996) reported that the presence of δ subunits inhibited neurosteroid modulation but not direct activation, of $GABA_A$ receptors. However, a recent study (Mihalek et al., 1999) demonstrated that mice lacking the $GABA_A$ receptor δ subunit had attenuated behavioral responses to systemic neurosteroid administration. This suggested an important role for the δ subunit either in the neurosteroid modulation of $GABA_A$ receptor currents or in the neural circuits relevant to the behavioral effects of neurosteroids. Approximately 30% of cerebellar $GABA_A$ receptors are thought to contain the δ subunit. δ mRNA is also found in the hippocampus and thalamus (Benke et al., 1991; Laurie et al., 1992a,b; McKernan and Whiting, 1996).

We used whole-cell and single-channel patch-clamp recordings and applied GABA using an ultra fast application system to

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investigate neurosteroid allosteric modulation of $GABA_A$ receptor currents in mammalian cells transiently transfected with recombinant $GABA_A$ receptors containing $\alpha1$ or $\alpha6$ with $\beta3$ and $\gamma2L$ or δ subunits.

MATERIALS AND METHODS

Expression of recombinant $GABA_A$ receptors. The cDNAs encoding rat $\alpha1$, $\alpha6$, $\beta3$, δ, and $\gamma2L$ $GABA_A$ receptor subunit subtypes were individually subcloned into the plasmid expression vector pCMVNeo. All subunits have been sequenced and are identical to published sequences. Human embryonic kidney cells (HEK293T; a gift from P. Connely, COR Therapeutics, San Francisco, CA) were maintained in DMEM, supplemented with 10% fetal bovine serum, at 37°C in 5% CO_2/95% air. Cells were transfected with 4 μg of each subunit plasmid along with 1–2 μg of pHOOK (Invitrogen, Carlsbad, CA) for immunomagnetic bead separation (Greenfield et al., 1997), using a modified calcium phosphate coprecipitation technique as described previously (Angelotti et al., 1993). The next day, cells were replated, and recordings were made 18–30 hr later.

Electrophysiology and drug application. Patch-clamp recordings were performed on transfected fibroblasts bathed in an external solution consisting of (in mM): NaCl 142, KCl 8, $MgCl_2$ 6, $CaCl_2$ 1, HEPES 10, glucose 10, pH 7.4, 325 mOsm). Electrodes were formed from soda lime (whole cell), thin-walled borosilicate (whole cell), or thick-walled borosilicate (excised patch) glass (World Precision Instruments, Pittsburgh, PA) with a Flaming Brown electrode puller (Sutter Instrument Co., San Rafael, CA). Electrodes had resistances of 0.8–8.0 MΩ when filled with an internal solution consisting of (in mM): KCl 153, $MgCl_2$ 1, MgATP 2, HEPES 10, EGTA 5, pH 7.3, 300 mOsm. Lower resistance electrodes were used for experiments in which cells were lifted from the recording dish (see Fig. 5). Higher resistance electrodes were used for single-channel recordings and were coated with Q-dope. The combination of internal and external solutions produced a chloride equilibrium potential near 0 mV. Unless stated otherwise, cells were voltage clamped at −10 to −75 mV using either an Axon 1D or a 200A amplifier (Axon Instruments, Foster City, CA). No voltage-dependent effects were observed in this range. Tetrahydrodeoxycorticosterone (THDOC) (Sigma, St. Louis, MO) was prepared as a 10 mM stock in dimethylsulfoxide (DMSO). THDOC was dissolved in external solution containing DMSO at a maximal final concentration of 0.1%. For most experiments, drugs were applied using a modified U-tube (Greenfield et al., 1997). For preapplication experiments, drugs were delivered (via gravity) to whole cells using a rapid perfusion system consisting of three-barrel square glass connected to a Warner Perfusion Fast-Step (Warner Instrument Corp., Hamden, CT). The glass was pulled to a final barrel size of ~250 μm. The solution exchange time was estimated routinely by stepping a dilute external solution across the open electrode tip to measure a liquid junction current. The 10–90% rise times for solution exchange were consistently ≤1–2 msec, although exchange was probably slower around cells. For single-channel experiments, drugs were applied either directly to the bath or via the multibarrel apparatus.

Analysis of currents. Whole-cell currents were low-pass filtered at 2–5 kHz, digitized at 10 kHz, and analyzed using the pCLAMP8 software suite (Axon Instruments). For concentration–response plots, peak currents evoked by GABA or THDOC at multiple concentrations were fitted to a sigmoidal function using a four-parameter logistic equation (sigmoidal concentration–response) with a variable slope. The equation used to fit the concentration–response relationship was $I = I_{(max)}/1 + 10^{(\mathrm{LogEC}50 - \mathrm{Log}_{drug})}$*Hill slope, where I was the peak current at a given GABA concentration, and $I_{(max)}$ was the maximal peak current. The desensitization and deactivation time courses of $GABA_A$ receptor currents elicited with the concentration jump technique were fit using the Levenberg-Marquardt least squares method with one or two or three component exponential functions of the form $\Sigma\, a_n\tau_n$, where n is the best number of exponential components, a is the relative amplitude of the component, and τ is the time constant. Additional components were accepted only if they significantly improved the fit, as determined by an F test on the sum of squared residuals. For comparison of deactivation time courses, a weighted summation of the fast and slow decay components ($a_f * \tau_f + a_s * \tau_s$) was used. Single-channel data were digitized at 20 kHz, filtered at 2 kHz via the internal Axon 200A amplifier filter, and stored on VHS videotape for analysis off-line. Stretches of single-channel activity were analyzed using the 50% threshold detection method of Fetchan 6.0 (pClamp 8.0). Overlapped openings and bursts were not included in the analysis. Although overlapping openings, indicating multiple channels, were observed in most patches, they would not affect the open duration histograms. Open duration histograms were generated and fitted using Interval5 software (Dr. Barry S. Pallotta, University of North Carolina, Chapel Hill, NC). The number of exponential functions required to describe the data was determined by a log-likelihood method (additional components were accepted if they significantly improved the fit). Events with durations <150 μsec (1.5 times the system dead time) were shown in the plots but were not considered by the fitting routine. Additional data reduction and filtering were implemented for figure display purposes only. Numerical data were expressed as mean ± SEM. Statistical significance, using Student's t test (two-tailed, paired, or unpaired as appropriate) was taken as $p < 0.05$.

RESULTS

Direct activation by THDOC depended on subunit composition

All constructs produced THDOC-sensitive currents in HEK293T cells. Cells were voltage clamped at −65 mV, and whole-cell currents were recorded in response to increasing concentrations of THDOC (Fig. 1*A*,*C*). The $GABA_A$ receptor isoforms exhibited different THDOC sensitivities (EC_{50} values) (Fig. 1*C*). Although there is little mechanistic information in this analysis, it is necessary to describe direct activation so that appropriate concentrations can be chosen for subsequent modulation experiments (see below). Additionally, we observed a subunit and subtype dependence of the direct effects of THDOC. $\alpha6$ subtype-containing receptors had lower EC_{50} values for THDOC activation than $\alpha1$ subtype-containing receptors ($p < 0.05$), similar to observations for GABA concentration–response curves (Fisher et al., 1997) (Fig. 1*C*, Table 1). The $\alpha1\beta3\gamma2L$ isoform was ~2.5-fold less sensitive to THDOC than the $\alpha6\beta3\gamma2L$ isoform. The $\alpha1\beta3\delta$ isoform was at least sixfold less sensitive to THDOC than the $\alpha6\beta3\delta$ isoform. Regarding the $\alpha1\beta3\delta$ receptor complex, a complete concentration–response curve could not be obtained because of high final DMSO concentration (>0.3%) with higher THDOC concentrations (>30 μM). The direct activation was first observed at 30–100 nM THDOC for all receptor isoforms. At higher concentrations of THDOC (>10 μM), a "rebound" current was observed on washout in all constructs. The rebound was more clearly evident under conditions of faster perfusion (Fig. 1*B*), which may explain why this effect was not reported previously. Maximum currents were significantly different between $\gamma2L$ and δ subunit-containing receptors ($p < 0.05$) (Table 1). THDOC elicited larger currents from $GABA_A$ receptors containing the $\gamma2L$ subunit than from receptors containing the δ subunit (Fig. 1*C*, Table 1). $\alpha1\beta3$ and $\alpha6\beta3$ isoforms showed current amplitudes intermediate between $\alpha\beta\delta$ and $\alpha\beta\gamma$ isoforms (Fig. 1*C*). For each isoform, maximum currents were in the same range of peak amplitudes when evoked by THDOC or GABA (data not shown).

THDOC produced increased modulation of $\alpha1\beta3\delta$ receptor currents

To evaluate the effect of subunit composition on THDOC modulation of $GABA_A$ receptor currents, increasing concentrations of THDOC were coapplied with an EC_{30} GABA concentration determined for each isoform. Low THDOC concentrations (<300 nM) were considered "modulatory" concentrations, because little or no direct activation was observed in this range in whole-cell recordings. Higher concentrations of THDOC resulted in more substantial direct activation of $GABA_A$ receptor currents. Neurosteroid modulation was observed for all isoforms

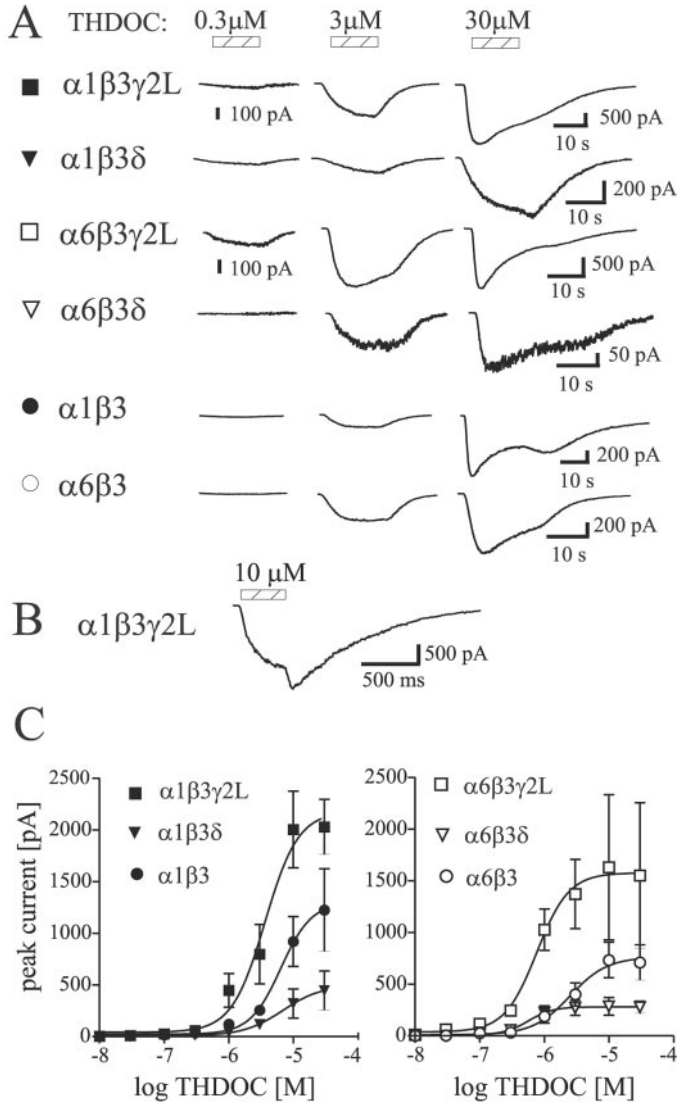

Figure 1. Direct activation of $GABA_A$ receptors by THDOC. *A*, Representative currents evoked by increasing THDOC concentrations for $\alpha1\beta3\gamma2L$ (■), $\alpha1\beta3\delta$ (▼), $\alpha6\beta3\gamma2L$ (□), $\alpha6\beta3\delta$ (▽), $\alpha1\beta3$ (●), and $\alpha6\beta3$ (○) $GABA_A$ receptor isoforms. The *hatched bars* indicate application of various THDOC concentrations. Note the small inflections in the currents after application of 30 μM THDOC. *B*, Concentration jump using 10 μM alphaxalone shows a rebound current more clearly because of faster solution exchange (see Materials and Methods). *C*, Concentration–response relations for direct activation by THDOC. Mean ± SEM current amplitudes are shown. The *left* and *right panels* show α1- and α6-containing isoforms, respectively. Smaller currents were observed for δ subunit-containing isoforms. Symbols are as in *A*. See Table 1 for fitted parameters.

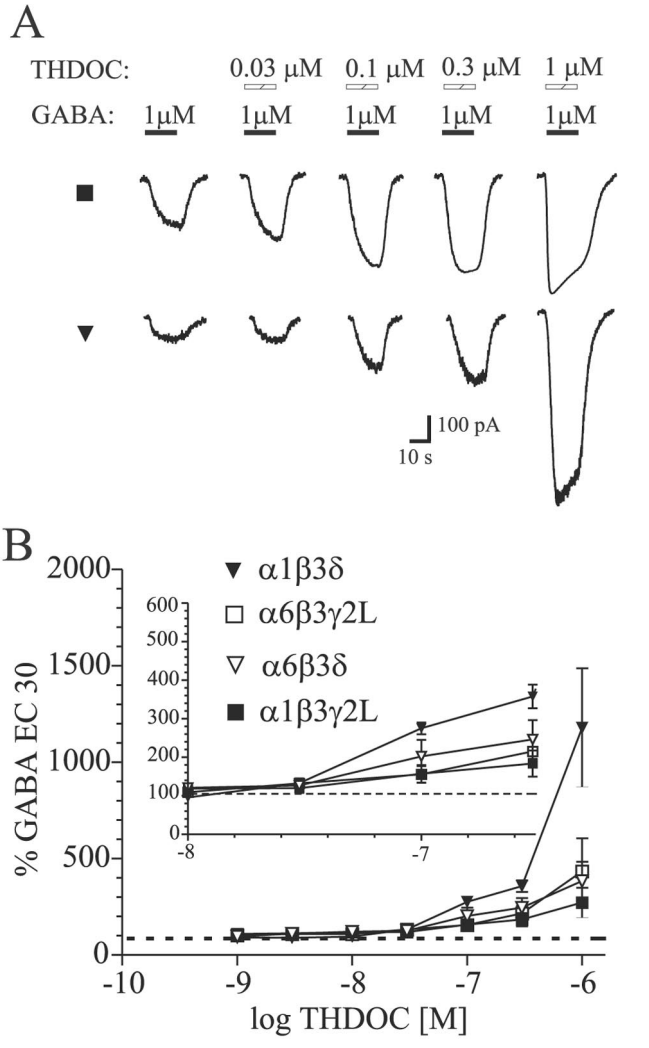

Figure 2. Modulation of submaximal GABA-evoked currents by THDOC coapplication. *A*, Current traces showing THDOC enhancement of EC_{30} GABA concentrations for $\alpha1\beta3\gamma2L$ (■) and $\alpha1\beta3\delta$ (▼) $GABA_A$ receptors. *Hatched bars* indicate THDOC application; *filled bars* indicate GABA application. *B*, Summary plot of THDOC enhancement of currents evoked by EC_{30} GABA concentration for each isoform. Data from lower THDOC concentrations are expanded in the *inset* for clarity. Significant potentiation was observed for THDOC concentrations of 100 nM and higher ($p < 0.05$).

tested (Fig. 2). For $\alpha1\beta3\delta$ receptors, the extent of the modulatory effect was more pronounced than for the other isoforms. For $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2L$ receptors, a significant difference in THDOC-induced potentiation was detected for concentrations of 100 nM, 300 nM, and 1 μM ($p < 0.05$). At concentrations below 100 nM, the modulatory effect of THDOC was not significant different among all isoforms tested. Replacement of δ with γ2L subunits reduced the apparent $GABA_A$ receptor sensitivity to THDOC potentiation. The α6 subtype-containing $GABA_A$ receptors were enhanced similarly by THDOC, whether the δ or γ2L subunit was present.

THDOC enhanced $GABA_A$ receptor currents without changing the GABA EC_{50}

Although concentration–response curves are empirical descriptions and do not specify any particular mechanism, the effect of modulators on the GABA concentration–response relation can provide insight into modulator mechanism(s) of action. Thus, complete GABA concentration–response curves with and without 30 nM THDOC were obtained for each isoform (Fig. 3). A THDOC concentration of 30 nM was chosen as a modulatory concentration because it was less than EC_5 for THDOC direct activation for each isoform (Fig. 1), thus minimizing the effect of direct activation. Furthermore, in each cell, 30 nM THDOC was applied alone to verify the minimal activation of current compared with GABA-evoked currents and to confirm that such currents, when present, were not significantly affecting the analysis. For all $GABA_A$ receptor isoforms tested, THDOC clearly enhanced the GABA-activated currents. Although the GABA

Table 1. Pharmacological properties of studied $GABA_A$ receptors

	THDOC				GABA			THDOC + GABA			
Isoform	*n*	EC_{50}	nH	I_{max} (pA)	*n*	EC_{50}	nH	*n*	EC_{50}	nH	Max (%)
$\alpha1\beta3\gamma2L$	3	1.9	1.4	2141 ± 200	4	2.3	1.4	6	2.8	1.5	128 ± 10
$\alpha1\beta3\delta$	4	9.5[a]	0.9	498 ± 150	4	5.8	1.0	4	6.6	1.1	265 ± 47*
$\alpha1\beta3$	3	6.7	1.1	1302 ± 360	3	3.0	2.0	3	1.7	1.6	113 ± 4
$\alpha6\beta3\gamma2L$	4	0.7	2.2	1588 ± 700	5	0.6	1.3	4	0.6	1.3	124 ± 6
$\alpha6\beta3\delta$	3	1.4	1.3	285 ± 60	4	0.6	1.1	5	0.4	1.3	145 ± 15
$\alpha6\beta3$	3	2.6	1.6	778 ± 130	3	0.6	1.2	3	1.2	1.1	152 ± 26

n indicates the number of cells tested. All EC_{50} values are in micromolar. nH is the hill coefficient. Max (%) indicates the maximum current observed with THDOC + GABA compared with maximum current observed with GABA alone.

* Significant difference from all other isoforms ($p < 0.05$).

[a] Indicates that full concentration–response curve could not be obtained because of high DMSO vehicle concentration.

EC_{50} values remained unchanged, the maximum currents were significantly enhanced for each isoform ($p < 0.05$) (Table 1). The extent of potentiation by THDOC was significantly greater for the $\alpha1\beta3\delta$ isoform (Fig. 3*C*) than for the $\alpha1\beta3\gamma2L$ isoform (Fig. 3*A*) and the $\alpha1\beta3\delta$ isoform (Fig. 3*E*) ($p < 0.05$). No significant difference in THDOC-induced potentiation was detected among $\alpha6\beta3\gamma2L$, $\alpha6\beta3\delta$, and $\alpha6\beta3$ receptors (Fig. 3*B*,*D*,*F*). Focusing on α subunit subtype dependence, the extent of potentiation by THDOC was significantly higher for $\alpha1\beta3\delta$ isoforms than for $\alpha6\beta3\delta$ isoforms ($p < 0.05$) and higher for $\alpha6\beta3$ than for $\alpha1\beta3$ isoforms ($p < 0.05$), whereas no significant difference was found between $GABA_A$ receptors containing $\alpha1\beta3\gamma2L$ and $\alpha6\beta3\gamma2L$ subunits.

Neurosteroid modulation changed polarity at high GABA concentration

To investigate further the differences in neurosteroid modulation of $\alpha1\beta3\gamma2L$ and $\alpha1\beta3\delta$ isoforms at high GABA concentration likely to occur at synapses, we coapplied increasing concentrations of THDOC with 1 mM GABA (Fig. 4*A*). We chose these two $GABA_A$ receptors because of the significant difference in their THDOC-induced modulation described earlier. Consistent with previous data from cerebellar granule neurons (Zhu and Vicini, 1997), at a saturating concentration of GABA, the polarity of THDOC modulation of $\alpha1\beta3\gamma2L$ receptors reversed (Fig. 4*B*): peak currents were inhibited by THDOC in a concentration-dependent manner. In contrast, $\alpha1\beta3\delta$ receptor currents were increasingly potentiated by THDOC concentrations (up to 30 μM) (Fig. 4*B*). Although direct activation of $GABA_A$ receptor currents occurred at high THDOC concentrations, the strong potentiation observed for $\alpha1\beta3\delta$ receptors could not be accounted for simply by increased direct activation (Fig. 5).

THDOC altered the macroscopic desensitization of $\alpha1\beta3\delta$ $GABA_A$ receptor currents

Having established a clear difference between modulation of $\alpha1\beta3\gamma2L$ and $\alpha1\beta3\delta$ currents by THDOC, we used the concentration jump technique to determine the effects on macroscopic kinetics using preapplication of THDOC (Figs. 5, 6). After obtaining a control response to GABA, cells were jumped from control solution to THDOC (1 μM) alone for preincubation of at least 1.5 sec, then to THDOC (1 μM) plus GABA (1 mM) for 4 sec, and then allowed to deactivate in the presence of THDOC (1 μM) alone. This allowed us to separate the effects of direct activation from modulation of the currents (because the THDOC was preapplied), and the rapid solution changes allowed better resolution of peak currents, desensitization, and deactivation. Also, the preapplication protocol ensured that the receptors were equilibrated with THDOC at the time of GABA application. THDOC (1 μM) reversibly potentiated both $\alpha1\beta3\delta$ (Fig. 5*A*) and $\alpha1\beta3\gamma2L$ (Fig. 5*B*) $GABA_A$ receptor currents elicited by low ($\sim EC_{30}$) concentrations of GABA. The mean enhancement was fourfold larger for $\alpha1\beta3\delta$ $GABA_A$ receptors ($\sim$1600%; $n = 6$) than for $\alpha1\beta3\gamma2L$ receptors ($\sim$400%; $n = 6$) ($p < 0.05$) (Fig. 6*A*, *left pair* of *bars*). Although the potentiated currents desensitized to a greater extent (measured as the percentage of current "lost" relative to peak current) than control currents for both isoforms, this effect was more pronounced for $\alpha1\beta3\gamma2L$ receptors. The left half of Figure 6*B* shows the subunit-dependent differences in desensitization extent in the presence of low concentration GABA with or without THDOC (compare *open* and *shaded bars* for each isoform). After washout of 1 μM GABA, the deactivation rate of THDOC-modulated currents was prolonged similarly for both isoforms ($\sim$250%) (Fig. 5*A*,*B*). The left pair of bars in Figure 6*C* shows THDOC-induced changes in the time constant of deactivation after washout of low concentration GABA.

The polarity of THDOC modulation of currents evoked by coapplication of a saturating (1 mM) concentration of GABA depended on subunit composition (Fig. 4). Similar results were observed during concentration jump experiments in which THDOC was preapplied. After a control response to GABA alone (1 mM) was obtained, cells were jumped into THDOC alone (1 μM) for a 1.5 sec preincubation followed by a 4 sec pulse of GABA + THDOC and then allowed to deactivate in the presence of THDOC. $\alpha1\beta3\delta$ $GABA_A$ receptors were enhanced by $\sim$800% ($n = 8$) (Fig. 5*C*), whereas $\alpha1\beta3\gamma2L$ $GABA_A$ receptors were inhibited $\sim$20% ($n = 4$) (Fig. 5*D*). The right half of Figure 6*A* summarizes this difference in THDOC modulation of peak current using saturating GABA concentration. The concentration jump experiments allowed resolution of subunit-dependent differences in the macroscopic kinetics of THDOC modulation as well. Specifically, THDOC (1 μM) substantially increased the rate and extent of desensitization of $\alpha1\beta3\delta$ currents (Figs. 5*C*, 6*B*). $\alpha1\beta3\delta$ receptors normally exhibit minimal desensitization, even in the presence of saturating (1 mM) GABA concentrations (Fig. 6*B*) (Saxena and Macdonald, 1994; Haas and Macdonald, 1999). In contrast, peak currents were inhibited, and the extent of macroscopic desensitization was unaltered by THDOC for $\alpha1\beta3\gamma2L$ receptor currents (Fig. 5*D*). Figure 6*B* (*right half*) summarizes the changes in extent of desensitization when 1 mM GABA was applied with or without THDOC (1 μM).

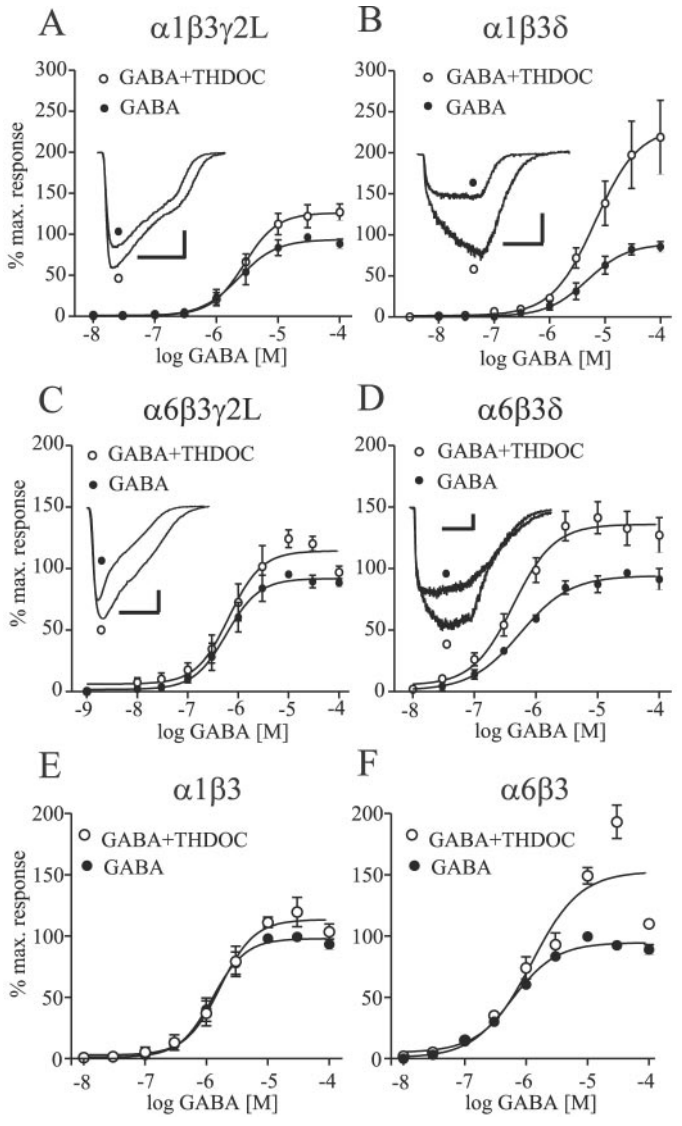

Figure 3. THDOC enhanced the maximal $GABA_A$ receptor currents without changing the GABA EC_{50}. *A–D*, GABA concentration–response curves were obtained in the absence (●) and presence (○) of 30 nM THDOC for α1β3γ2L (*A*), α1β3δ (*B*), α6β3γ2L (*C*), α6β3δ (*D*), α1β3 (*E*), and α6β3 (*F*) isoforms. Representative maximal GABA currents without (●) and with (○) THDOC coapplication are shown in the *inset* of *A–D*. For the GABA plus THDOC curves, the currents were normalized to the amplitude of a 100 μM GABA test pulse obtained from the same cell. Fitted parameters are given in Table 1.

Furthermore, the time course of desensitization of α1β3γ2L receptor currents evoked by 1 mM GABA was fitted best with the sum of three exponential functions with similar rate constants in the presence or absence of THDOC (Fib. 6*D*, *left portion*, compare *open* and *shaded bars*). The rate of desensitization could not be measured accurately for α1β3δ receptors during 4 sec pulses (because the time constant was much longer than the application duration). However, in the presence of THDOC, the weighted desensitization time constant decreased to ~2 sec (Fig. 6*D*). Although current deactivation after washout of 1 mM GABA was prolonged by THDOC (1 μM) for both isoforms, the relative increase was smaller for α1β3γ2L $GABA_A$ receptors ($p < 0.05$) (Fig. 6*C*, *right pair* of *bars*).

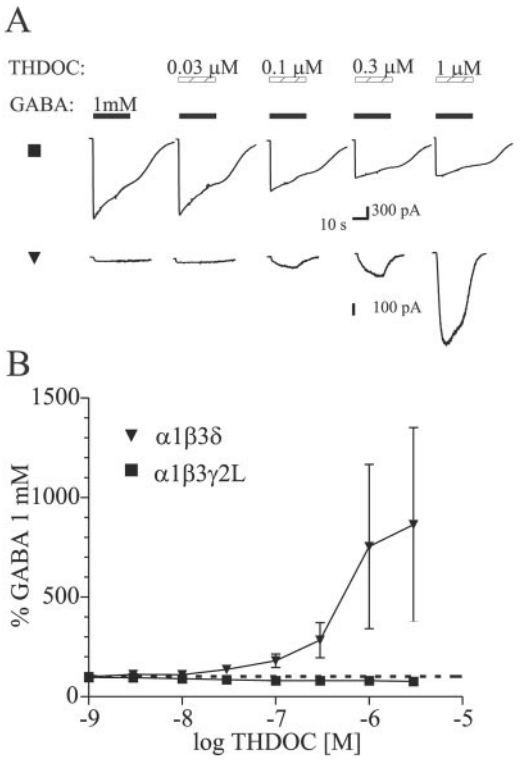

Figure 4. Modulation of maximal GABA-evoked currents by THDOC coapplication. *A*, Increasing concentrations of THDOC were coapplied with 1 mM GABA to α1β3γ2L (■) and α1β3δ (▼) $GABA_A$ receptors. Representative traces are shown for each isoform. *Hatched bars* indicate THDOC application; *filled bars* indicate GABA application. *B*, Summary of THDOC modulation, showing increasing enhancement for α1β3δ receptors and inhibition for α1β3γ2L receptors. The *dashed line* indicates 100% of control (1 mM GABA alone) current amplitude. *Symbols* indicate the mean ± SEM responses of four cells for each isoform.

THDOC introduced a third, longer open state for α1β3δ $GABA_A$ receptors

Single-channel recordings were obtained from α1β3δ receptors to investigate the basis for the large change in efficacy produced by THDOC. Consistent with our previous reports (Fisher and Macdonald, 1997; Haas and Macdonald, 1999), α1β3δ $GABA_A$ receptor single-channel openings evoked during steady-state application of 1 mM GABA were brief (Fig. 7*A1*), with a mean open duration of 0.445 ± 0.026 msec (Table 2). The distribution of open durations was best described by the sum of two exponential functions with time constants of ~300 μsec and ~1 msec (Fig. 7*A2*). Coapplication of 1 μM THDOC increased the mean channel opening duration. The distribution of open durations required a third exponential function to account for the longer openings, with a time constant of 5.94 ± 0.98 msec and relative area of 9.8 ± 2.9%. Although the shortest exponential function had a similar time constant, the second time constant and its relative area were increased significantly compared with the openings evoked by GABA alone ($p < 0.05$) (Table 2). Because THDOC (1 μM) can directly activate $GABA_A$ receptor currents, we also measured single-channel currents from α1β3δ $GABA_A$ receptors in the presence of THDOC (1 μM) alone. The mean open duration was not different from that observed with GABA alone, and the first two open durations were also unchanged in terms of time constant and relative area (Table 2). However, a third open state with small relative area (4.0 ± 1.6%) was required to fit the distribu-

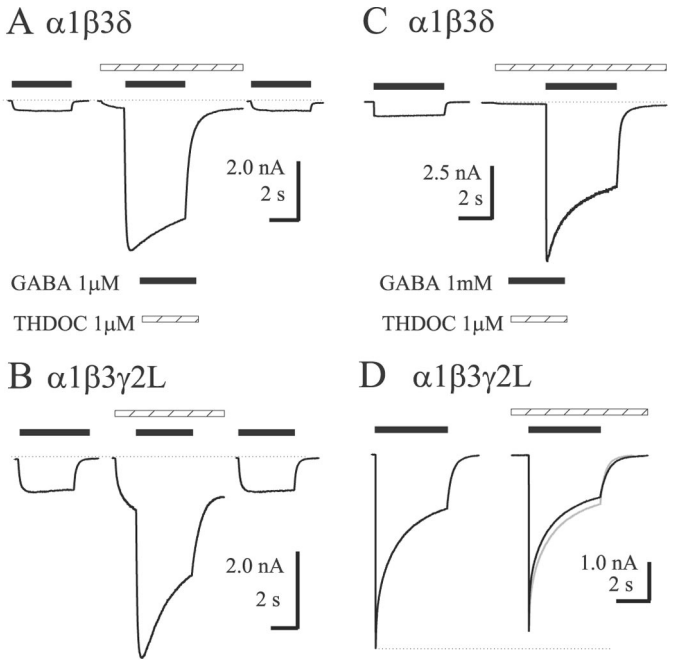

Figure 5. Kinetics and polarity of THDOC modulation depend on subunit composition and GABA concentration. THDOC (1 μM) was preapplied with 1 μM GABA (*A*, *B*) and 1 mM GABA (*C*, *D*) using the concentration jump technique. *Hatched bars* indicate THDOC application; *filled bars* indicate GABA application. For the rapidly desensitizing $\alpha1\beta3\gamma2L$ receptors, cells were lifted from the recording dish to increase resolution of the peak currents. Direct activation was observed during the preapplication for both isoforms, with greater relative currents evoked from $\alpha1\beta3\gamma2L$. Greater enhancement of 1 μM GABA currents was observed for $\alpha1\beta3\delta$ receptors, although both isoforms showed slightly increased desensitization and prolonged deactivation (*A*, *B*). With 1 mM GABA, $\alpha1\beta3\delta$ receptors were enhanced substantially and pronounced desensitization was observed (*C*), whereas $\alpha1\beta3\gamma2L$ receptors were slightly inhibited (*D*). The *control trace* in *D* was normalized and overlaid in *gray* to show the minimal effect on apparent desensitization. The *dashed line* emphasizes the decreased peak current in the presence of THDOC.

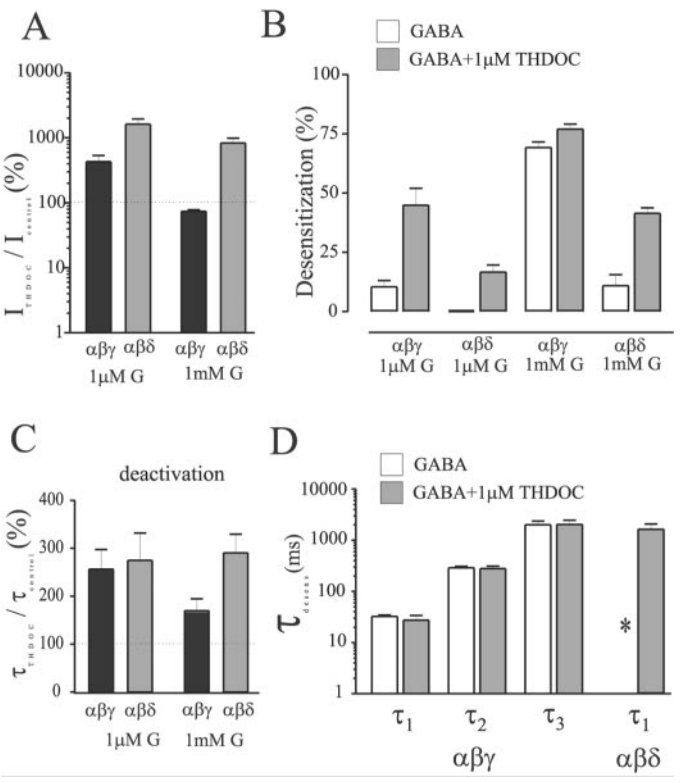

Figure 6. Summary of THDOC effects on peak currents, desensitization, and deactivation. *A*, Effect of THDOC (1 μM) on currents evoked by 1 μM and 1 mM GABA for $\alpha1\beta3\delta$ (*gray bars*) and $\alpha1\beta3\gamma2L$ (*black bars*) $GABA_A$ receptors. Values are expressed as a percentage of current evoked by GABA alone for each cell. Note the logarithmic axis. The *dashed line* indicates 100% of control current amplitude. *B*, Percentage increase in the weighted time constant of deactivation for the same conditions as in *A*. The *dashed line* indicates 100% of control deactivation. *C*, Extent of desensitization observed with 1 mM GABA alone (*white bars*) or 1 mM GABA + 1 μM THDOC (*gray bars*) for $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2L$ $GABA_A$ receptors. Data are expressed as the percentage of peak current lost during a 4 sec application of GABA or GABA + THDOC. *D*, The rates of desensitization during a 4 sec pulse of 1 mM GABA for $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2L$ $GABA_A$ receptors in the absence (*white bars*) and presence of preapplied 1 μM THDOC. $\alpha1\beta3\gamma2L$ $GABA_A$ receptor responses were fitted best by three exponentials with similar time constants and relative areas (data not shown) whether or not THDOC was present. Although the small amount of desensitization observed for $\alpha1\beta3\delta$ in the presence of GABA alone was not well fitted (*, the time constant was longer than the pulse duration), the pronounced desensitization observed in the presence of THDOC had a weighted time constant ~2 sec. The data are from four to eight cells per condition.

tion. The longer open state in the presence of THDOC alone accounted for 12.4% of the charge passed, whereas the longer open state in the presence of both drugs accounted for 39.0% of the charge passed. Although neurosteroids have been reported to increase the frequency of channel openings, we did not consider changes in open frequency because of the confounding appearance of desensitization in the presence of THDOC macroscopically (Fig. 5). At the single-channel level, quiescent periods rarely observed with GABA alone would decrease overall opening frequency in the presence of GABA and THDOC for $\alpha1\beta3\delta$ $GABA_A$ receptor channels.

DISCUSSION

$GABA_A$ receptors are targets for CNS actions of neurosteroids. Our results demonstrated a novel subunit dependence of neurosteroid action. Specifically, receptors containing the δ subunit were preferentially enhanced by the neurosteroid THDOC. THDOC affected both the single-channel gating kinetics and the macroscopic desensitization of $\alpha1\beta3\delta$ $GABA_A$ receptor channels. These findings may be the basis for the attenuated neurosteroid sensitivity in mice lacking the δ subunit (Mihalek et al., 1999). Additionally, our results suggest the importance of GABA concentration for THDOC modulation of receptors containing the γ2 subunit.

It is believed that neurosteroids, like barbiturates, exert their action on $GABA_A$ receptors via two distinct binding sites (Majewska et al., 1986; Gee et al., 1988; Turner et al., 1989; Lambert et al., 1995, 1996; Zorumski et al., 1998; Park-Chung et al., 1999). Low (nanomolar) concentrations of neurosteroids allosterically enhance GABA-mediated currents, whereas higher (micromolar) concentrations directly activate $GABA_A$ receptors. We observed a rebound current on washout of THDOC for concentrations ≥10 μM in all tested $GABA_A$ receptor isoforms. This phenomenon may indicate a third binding site, presumably within the channel pore, that produces a low-affinity open-channel block similar to that observed for barbiturates.

Although no clear subunit specificity has been demonstrated for neurosteroid modulation of $GABA_A$ receptor currents as there has been for benzodiazepine modulation of $GABA_A$ recep-

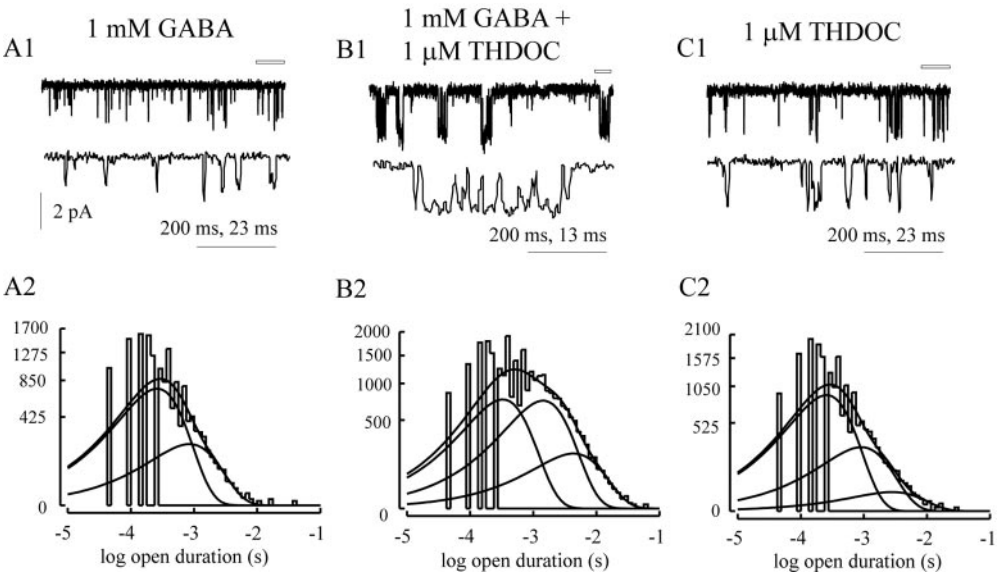

Figure 7. THDOC enhanced single-channel open duration in $\alpha1\beta3\delta$ $GABA_A$ receptor single channels. Representative $\alpha1\beta3\delta$ $GABA_A$ receptor single-channel currents evoked by 1 mM GABA alone (*A1*), 1 mM GABA + 1 M THDOC (*B1*), and 1 M THDOC alone (*C1*). A portion of the *top trace* in *each panel* is expanded in the trace directly beneath it (indicated by the *open bar*). The *traces* in *A1* and *B1* are from the same patch. The larger scale factor applies to the *top traces*. *A2*, *B2*, and *C2* are the open duration histograms for all patches obtained for each condition (n = 3, 5, and 3 respectively). *Superimposed lines* are the fitted exponential functions describing the distributions.

Table 2. Single-channel opening properties for $\alpha1\beta3\delta$ $GABA_A$ receptors

Drug	$\tau1$	A1	$\tau2$	A2	$\tau3$	A3	Mean	n
1 mM GABA	0.31 ± 0.02	0.81 ± 0.06	1.02 ± 0.09	0.19 ± 0.06			0.444 ± 0.026	3
1 mM GABA + 1 μM THDOC	0.357 ± 0.02	0.43 ± 0.04	1.822 ± 0.20	4.47 ± 0.03	5.94 ± 0.98	0.10 ± 0.03	1.52 ± 0.15	5
1 μM THDOC	0.29 ± 0.02	0.72 ± 0.02	1.00 ± 0.23	0.24 ± 0.01	3.94 ± 1.70	0.04 ± 0.02	0.535 ± 0.054	3

The open duration histograms were fitted with multiple exponential functions, where τ is the time constant and A is the relative area.

tor currents (Lambert et al., 1995), the α and γ subunits in $GABA_A$ receptors have some influence on the EC_{50} and efficacy of modulation by neurosteroids such as THDOC that act as positive modulators (Puia et al., 1993). Puia et al. (1990) and Zhu et al. (1996) reported no subunit-dependent differences in the sensitivity to THDOC activation or amplitude of THDOC-evoked currents among different $GABA_A$ receptor isoforms. However, our results indicate that the $\alpha1$ and $\alpha6$ subtypes conferred different EC_{50} values for THDOC, similar to the difference reported for GABA (Fisher et al., 1997). THDOC and GABA were equally effective as agonists for all studied $GABA_A$ receptors, as indicated by peak currents obtained in whole-cell recordings. However, the maximum current amplitudes produced by THDOC and GABA were larger for γ than for δ subunit-containing receptors, which might be related to differences in expression efficiency or intrinsic gating efficacy. This difference has been reported for currents evoked by GABA as well (Fisher and Macdonald, 1997; Haas and Macdonald, 1999). However, the basis for this difference remains unclear.

Zhu et al. (1996) reported that incorporation of the δ subunit inhibited the modulatory (but not the direct) effect of THDOC on $GABA_A$ receptor currents. In contrast, we found a significant neurosteroid potentiation in all tested receptors, and $\alpha1\beta3\delta$ receptors were potentiated more than $\alpha1\beta3\gamma2L$ and $\alpha1\beta3$ receptors. Replacement of $GABA_A$ receptor $\alpha1$ with $\alpha6$ subtypes in $\alpha\beta\delta$ receptors decreased the extent of THDOC potentiation, although THDOC enhanced both $\alpha1\beta3\gamma2L$ and $\alpha6\beta3\gamma2L$ $GABA_A$ receptor currents to similar extents. The latter finding contrasts with previous reports showing a decrease in steroid sensitivity with $\alpha6$ compared with $\alpha1$ in $\alpha\beta\gamma$ combinations (Puia et al., 1993; Zhu et al., 1996). Neurosteroid modulation of $\alpha1\beta3\delta$ receptor currents was greater than that of $\alpha6\beta3\delta$ and $\alpha1\beta3\gamma2L$ $GABA_A$ receptor currents. However, dentate granule cells become less sensitive to THDOC with developmental progression (Cooper et al., 1999), despite an increase in δ subunit expression in this brain region (Laurie et al., 1992a). This may be related to variable steroid modulation when δ subunits coassemble with α subtypes other than $\alpha1$, or when γ and δ subunits are present in the same receptor. Additionally, it is possible that a neuronal environment alters the sensitivity of $\alpha\beta\delta$ $GABA_A$ receptors. In fact, by combining electrophysiological recordings and single-cell PCR techniques, Zhu and Vicini (1996) observed an inverse relation between the presence of δ subunit mRNA and neurosteroid potentiation in cultured cerebellar granule cells. Also, there is evidence for a phosphorylation dependence of allopregnanalone modulation of GABAergic IPSCs in the hypothalamus (Fancsik et al., 2000). Such post-translational receptor modifications may contribute to potential differences between neuronal preparations and recombinant systems, among other cellular processes. Nevertheless, our results indicated that δ subunit-containing $GABA_A$ receptors are clearly enhanced by neurosteroids, particularly in combination with the $\alpha1$ subunit subtype, suggesting a critical role for the δ subunit in the assembly of neurosteroid-sensitive $GABA_A$ receptors. This observation is strengthened by the small degree of potentiation observed in $\alpha1\beta3$ $GABA_A$ receptors. The enhanced THDOC sensitivity of $\alpha\beta\delta$ isoforms was in agreement with a recent report by Mihalek et al. (1999), who found that the absence of the δ subunit resulted in a significant decrease in the sensitivity to neurosteroids. However, the precise mechanism by which the δ subunit knock-out attenuated neurosteroid effects awaits further study. It should be noted that $GABA_A$ receptors containing both $\gamma2$ and δ subunits may be

present *in vivo*. Whether the presence of a δ subunit would have a "dominant" effect on neurosteroid modulation remains unknown. Our data also support a recent study showing preferential steroid enhancement of $\alpha4\beta3\delta$ over $\alpha4\beta3\gamma2$ $GABA_A$ receptors (Adkins et al., 2001).

We have shown previously that incorporation of the δ subunit abolishes fast desensitization and reduces the overall rate and extent of desensitization (Saxena and Macdonald, 1994; Haas and Macdonald, 1999; Bianchi et al., 2001). Even at saturating (1 mM) GABA concentrations, minimal desensitization and relatively fast deactivation are observed. However, in this study we found pronounced desensitization in $\alpha1\beta3\delta$ $GABA_A$ receptors in the presence of 1 μM THDOC. This was probably not caused by open channel block because single-channel open durations were longer, not shorter as would be expected with such a mechanism. The apparent desensitization was accompanied by substantially prolonged deactivation. Although this finding is consistent with the suggested role of desensitized states in the duration of current deactivation (Jones and Westbrook, 1995, 1996), an increase in open frequency and duration could also prolong deactivation. In fact, single-channel recordings revealed that THDOC enhanced current through $\alpha1\beta3\delta$ $GABA_A$ receptors, at least through an increase in mean open duration. Steroids were reported previously to increase open duration (Mistry and Cottrell, 1990) and increase frequency as well as duration (Twyman and Macdonald, 1992) in native $GABA_A$ receptor single channels obtained from mouse spinal neurons, although it is unlikely that these channels contained the δ subunit. We did not analyze open frequency because of the introduction of desensitized states in $\alpha1\beta3\delta$ $GABA_A$ receptors. Periods of desensitization, however, would not confound the analysis of open durations. Although saturating GABA concentrations evoked single-channel openings best described by two exponential functions, a third longer duration open state was observed in the presence of THDOC. We propose that THDOC alters the intrinsic gating behavior of $\alpha1\beta3\delta$ $GABA_A$ receptors in at least two ways: (1) by allowing entry into otherwise unavailable desensitized states and (2) by increasing the gating efficacy via changes in opening duration. This change was attributable to an increase in the time constant of the second open state (1.8 compared with 1.0 msec; $p < 0.05$), a change in the relative proportion of the first and second open states, and the introduction of an additional longer open state (Table 2). Note that the modulation of single-channel gating was measured at steady state, so no direct comparisons (in terms of the magnitude of THDOC modulation) can be made with the transient applications performed on whole cells. Although the binding of GABA alone appears insufficient to allow entry into the longer open state, the concomitant binding of THDOC favors transitions to the longer state. Interestingly, THDOC alone (1 μM) activated single-channel events that were well described by three exponential functions, suggesting that neurosteroid binding alone may be sufficient to favor longer openings, although these longer openings were shorter and less frequent than the longer openings observed in the presence of both drugs. Thus it is unlikely that receptors bound only by THDOC contributed to the distinct gating behavior observed in the presence of both drugs. Additionally, the concentration of GABA (1 mM) was more than two orders of magnitude above the functional EC_{50} value for the $\alpha1\beta3\delta$ combination, resulting in near-saturating occupancy of the GABA binding sites at steady state. It remains unclear whether this effect is unique to the $\alpha1\beta3\delta$ isoform. However, given the weakly inhibited amplitude and similar desensitization time course in the presence of THDOC, it is unlikely that such dramatic effects on single-channel gating would be observed for $\alpha1\beta3\gamma2L$ $GABA_A$ receptor single channels.

The action of THDOC on channel gating kinetics may be analogous to the effects of barbiturates, which have been shown to prolong native (likely $\alpha\beta\gamma$) $GABA_A$ receptor single-channel mean open time by shifting the relative distribution of existing open durations, and similar effects were observed for neurosteroids (Twyman et al., 1989; Twyman and Macdonald, 1992). However, because the longer open state is not significant in the presence of GABA alone, we cannot explicitly demonstrate that this state can be accessed "naturally" by receptors bound by GABA alone. It is unknown whether barbiturates or other modulators can alter the gating behavior of $\alpha\beta\delta$ $GABA_A$ receptors in a manner similar to THDOC.

For $\alpha1\beta3\gamma2L$ $GABA_A$ receptors, modulation of maximal currents by 1 μM THDOC was similar to that reported for cerebellar granule neurons (Zhu and Vicini, 1997) in that peak currents were depressed and deactivation was prolonged, despite no change in the time course of desensitization. This effect contrasts with neurosteroid potentiation of currents activated by low GABA concentration in cerebellar granule neurons (Zhu and Vicini, 1997) and $\alpha1\beta3\gamma2L$ $GABA_A$ receptors (this study). Although nucleated patches obtained from cerebellar granule neurons in that study were likely to contain extrasynaptic receptors [which Nusser et al. (1998) reported to contain the δ subunit], our data are consistent with γ2 subunit- but not δ subunit-containing isoforms being predominant in that preparation.

In the cerebellum, δ subunit-containing receptors are thought to be extrasynaptic, whereas synaptic receptors are thought to preferentially contain the γ2 subunit (Nusser et al., 1998). However, Mihalek et al. (1999) suggested δ subunit involvement in normal synaptic transmission in the dentate gyrus. Although the synaptic concentration of GABA remains controversial, our results and those of others (Harrison et al., 1987; Zhu and Vicini, 1997) suggest that neurosteroids may modulate IPSCs in at least two ways: (1) by changing the peak current (either positively or negatively, depending on the concentration of both GABA and the neurosteroid) and (2) by prolonging the duration of the IPSC (by slowing deactivation) independent of the GABA concentration. For extrasynaptic $\alpha\beta\delta$ isoforms, neurosteroids may increase basal levels of inhibition by increasing the response to ambient GABA levels. It is also possible that modulation of neuronal circuits necessary for neurosteroid effects depend (either directly or indirectly) on $GABA_A$ receptors containing the δ subunit.

In summary, our results showed that the subunit composition of $GABA_A$ receptors is an important determinant of the neurosteroid modulation of $GABA_A$ receptor activity. Although the precise contribution of $\alpha\beta\delta$ $GABA_A$ receptor combinations toward the *in vivo* effects of neurosteroids remains to be elucidated, the enhanced potentiation of $\alpha1\beta3\delta$ $GABA_A$ receptors may indicate a critical role for this isoform.

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