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Neurosteroids Shift Partial Agonist Activation of $GABA_A$ Receptor Channels from Low- to High-Efficacy Gating Patterns

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Although GABA activates synaptic ($\alpha\beta\gamma$) $GABA_A$ receptors with high efficacy, partial agonist activation of $\alpha\beta\gamma$ isoforms and GABA activation of the primary extrasynaptic ($\alpha\beta\delta$) $GABA_A$ receptors are limited to low-efficacy activity, characterized by minimal desensitization and brief openings. The unusual sensitivity of $\alpha\beta\delta$ receptor channels to neurosteroid modulation prompted investigation of whether this high sensitivity was dependent on the δ subunit or the low-efficacy channel function that it confers. We show that the isoform specificity ($\alpha\beta\delta > \alpha\beta\gamma$) of neurosteroid modulation could be reversed by conditions that reversed isoform-specific activity modes, including the use of β-alanine to achieve increased efficacy with $\alpha\beta\delta$ receptors and taurine to render $\alpha\beta\gamma$ receptors low efficacy. We suggest that neurosteroids preferentially enhance low-efficacy $GABA_A$ receptor activity independent of subunit composition. Allosteric conversion of partial to full agonism may be a general mechanism for reversibly scaling the efficacy of $GABA_A$ receptors to endogenous partial agonists.

Key words: **$GABA_A$ receptor; neurosteroid; modal gating; desensitization; δ subunit; taurine; β-alanine**

Introduction

Many $GABA_A$ receptor modulators exhibit clear subunit selectivity (Olsen and Macdonald, 2002). $GABA_A$ receptor pharmacological studies have focused on "structural" determinants of modulators, such as the subunit dependence of benzodiazepines (Sigel and Buhr, 1997) and the proposed "transduction" element at the outer mouth of the second transmembrane domain (TM2) (Wingrove et al., 1994; Stevenson et al., 1995; Halliwell et al., 1999; Thompson et al., 1999). "Knock-in" mutations of the benzodiazepine binding site emphasized the importance of characterizing the isoform preferences of allosteric modulators (Rudolph et al., 1999; Low et al., 2000; McKernan et al., 2000).

In addition to the rich isoform-specific pharmacology of $GABA_A$ receptor channels, there is clear evidence that biophysical properties, such as desensitization and gating efficacy, also depend on subunit composition (Fisher and Macdonald, 1997; Haas and Macdonald, 1999). One of the potential confounding factors associated with investigating subunit-specific pharmacology is that "functional" differences among isoforms, as opposed to simply the presence or absence of modulator binding sites, may play a significant role in determining pharmacological profiles. Therefore, the observation of subunit-dependent modulation might in some cases be an epiphenomenon, in which the action of the modulator was dependent on a difference in a functional property (not a strictly structural one, per se) that was itself subunit-dependent.

The idea that allosterism could also depend on functional differences would offer yet another mechanism for specificity among isoforms, even if the binding site and coupling machinery of the modulator (the often sited basis for subunit specificity) were present in every isoform. It is even more intriguing to consider the potential plasticity of allosteric modulation given the observation of agonist-dependent functional properties of $GABA_A$ receptors. For example, partial agonists and so-called nondesensitizing agonists have been characterized, and some of these compounds (such as taurine) are present in the brain and may be endogenous ligands for $GABA_A$ receptors (Sakai et al., 1985; Lerma et al., 1986; Huxtable, 1989). Although the physiological relevance of endogenous partial agonists remains poorly understood, the possibility of reversible augmentation of partial agonist efficacy by endogenous modulators raises the interesting possibility of plasticity at the level of agonist-dependent gating.

One class of compounds that shows a clear $GABA_A$ receptor isoform preference is the neurosteroids (Mellon and Griffin, 2002), which exert their actions in the CNS in part through interaction with synaptic $GABA_A$ receptors (Harrison et al., 1987; Lambert et al., 1995; Cooper et al., 1999; Fancsik et al., 2000). The $GABA_A$ receptor δ subunit is of particular importance for behavioral responses to neurosteroids (Mihalek et al., 1999), and the endogenous neurosteroid tetrahydrodeoxycorticosterone (THDOC) preferentially enhanced $\alpha\beta\delta$ over $\alpha\beta\gamma$ receptors (Adkins et al., 2001; Brown et al., 2002; Wohlfarth et al., 2002). In-

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terestingly, THDOC modulation produced substantially larger currents than the maximal currents produced by GABA alone for $\alpha1\beta3\delta$ (but not $\alpha1\beta3\gamma2L$) receptors, suggesting that the low-efficacy activity of $\alpha\beta\delta$ receptors (Fisher and Macdonald, 1997) could be overcome by neurosteroids (Wohlfarth et al., 2002). The present study was designed to determine whether the enhanced neurosteroid actions depended on the presence of the δ subunit or the associated functional properties of minimal desensitization and low-efficacy gating.

Materials and Methods

Expression of recombinant $GABA_A$ receptors. Human embryonic kidney 293T (HEK293T) cells (a gift from P. Connely, COR Therapeutics, San Francisco, CA) were maintained in DMEM and supplemented with 10% fetal bovine serum at 37°C in 5% CO_2/95% air. Cells were transiently transfected with 4 μg each of α1 and β3 subunits, together with either a δ, γ2L, or mutated δ subunit (all subcloned into the **pCMVneo** expression vector), using the calcium phosphate precipitation technique (Angelotti et al., 1993). Point mutants were generated using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Cotransfection of the pHook plasmid (Invitrogen, Carlsbad, CA) enables selection of transfected cells by immunomagnetic bead separation 24 hr later (Greenfield et al., 1997). The next day, whole-cell patch-clamp recordings were performed at room temperature.

Electrophysiology and drug application. Patch-clamp recordings were performed on transfected fibroblasts bathed in an external solution consisting of the following (in mM): 142 NaCl, 8 KCl, 6 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.4, 325 mOsm. Low-resistance electrodes (0.8–1.5 MΩ; World Precision Instruments, Pittsburgh, PA) were pulled with a Flaming Brown electrode puller (Sutter Instruments, San Rafael, CA) and fire-polished. The internal solution consisted of the following (in mM): 153 KCl, 1 $MgCl_2$, 2 MgATP, 10 HEPES, 5 EGTA, pH 7.3, 300 mOsm. The combination of internal and external solutions produced a chloride equilibrium potential near 0 mV. Patch-clamped cells were gently lifted from the recording dish to increase solution-exchange efficiency. Cells were voltage-clamped at −10 to −50 mV, and no voltage-dependent effects of desensitization or neurosteroid modulation were observed in this range. For experiments involving excised patches, thick-walled borosilicate glass was used with resistances of 5–15 MΩ, and cells were plated on collagen-treated culture dishes.

THDOC (Sigma, St. Louis, MO) was prepared as a 10 mM stock in dimethylsulfoxide (DMSO) and kept frozen. The THDOC stock was dissolved in external solution containing DMSO at a final concentration of 0.1%. piperidine-4-sulfonic acid (P4S) (Sigma) prepared as a 100 mM stock in water. GABA was prepared as a 1 M stock in water. Drugs were applied via gravity using a rapid perfusion apparatus (Warner Instruments, Hamden, CT) connected to multibarrelled square glass tubing pulled to a final barrel size of ~250 μm. Solution exchange time measured with an open electrode tip was 0.3–1.5 msec, depending on the flow rate (with faster range used for excised patch experiments), although slower exchange probably occurred around whole cells.

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, Foster City, CA). To avoid underestimating the effects of THDOC on peak current amplitude resulting from current rundown, control measurements (GABA alone) were made before and after THDOC application, and the average response was used. For THDOC modulation, the small "direct" activation current observed during the preapplication period was subtracted from the peak current in the presence of GABA and THDOC. 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, two, or three component exponential functions of the form $\Sigma\, a_n e^{(-t/\tau n)}$, where n is the best number of exponential components, a is the relative amplitude of the component, t is time, 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 ($a_f \cdot \tau_f + a_s \cdot \tau_s$) was used. Single-channel data were digitized at 20 kHz, filtered at 2 kHz via the internal Axon 200A (Axon Instruments) amplifier filter, and stored on VHS videotape for off-line analysis. 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. Events with durations <150 μsec (1.5 times the system dead time) were shown in the histogram but were not considered in the fitting routine. Logarithmic binning was used as described previously (Haas and Macdonald, 1999) and fitted with a maximum likelihood routine by the Interval5 software (Dr. Barry Pallotta, University of North Carolina, Chapel Hill, NC). The number of exponential functions required to fit the distributions was incremented until additional exponentials failed to significantly improve the fit. Data reduction was implemented for figure display purposes only. Numerical data were expressed as mean ± SEM. Statistical significance using Student's t test (paired or unpaired, as appropriate) was taken as $p < 0.05$. All data sets were normally distributed.

Results

THDOC preferentially enhanced $\alpha1\beta3\delta$ over $\alpha1\beta3\gamma2L$ $GABA_A$ receptor currents

The neurosteroid THDOC has been shown to preferentially enhance $GABA_A$ receptors containing the δ subunit over those containing the γ2L subunit (Adkins et al., 2001; Brown et al., 2002; Wohlfarth et al., 2002). This effect is shown in Figure 1. Currents evoked by a saturating 1 mM GABA concentration were compared with and without pre-applied THDOC (1 μM; 2–3 sec) for $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2L$ receptors expressed in HEK293T cells (see Materials and Methods). $\alpha1\beta3\gamma2L$ receptor currents desensitized rapidly and extensively during a 6 sec application of GABA (1 mM) (Fig. 1*A*, left). In the presence of pre-applied THDOC (1 μM), peak currents were slightly inhibited (92.6 ± 9.9% of control amplitude; $n = 7$), although this was not significant (Fig. 1*C*), which is consistent with previous studies of neurons (Le Foll et al., 1997; Zhu and Vicini, 1997). Macroscopic desensitization in the presence of THDOC tended to be slightly faster, although this too was variable and neither the rates nor the extents of desensitization differed significantly from control values (Fig. 1*D*; Table 1). $\alpha1\beta3\delta$ receptor currents were substantially and reversibly (data not shown) enhanced by pre-applied THDOC (1290 ± 163%; $n = 8$) (Fig. 1*B*,*C*). Although this isoform exhibited minimal, slow desensitization even during saturating (1 mM) GABA application (Haas and Macdonald, 1999) (Table 1), THDOC-modulated currents showed pronounced desensitization during the 6 sec of GABA exposure (57.8 ± 2.6%) (Fig. 1*B*,*D*), which is in agreement with our previous results (Bianchi et al., 2002; Wohlfarth et al., 2002). In most cases, this increased desensitization was well described by two exponential functions, with time constants similar to the intermediate and slow time constants fitted to the triphasic desensitization of $\alpha1\beta3\gamma2L$ receptor currents evoked by 1 mM GABA (Table 1). The rate of current deactivation was prolonged by THDOC for both isoforms (Table 1). Clearly, THDOC enhanced $\alpha1\beta3\delta$ currents beyond the maximal currents evoked by a saturating concentration of GABA alone, and we showed previously that this was explained in part by the introduction of a longer duration, third, open state. In contrast, THDOC failed to increase the amplitude of $\alpha1\beta3\gamma2L$ receptor currents evoked by saturating GABA (1 mM). However, neurosteroids are well known to enhance submaximal $\alpha\beta\gamma$ receptor currents, and thus they clearly bind to the receptor (Puia et al., 1990; Lan et al., 1991; Adkins et al., 2001; Wohlfarth et al., 2002). Although this apparent GABA concentration dependence was similar to modulation by benzodiazepines (that only enhance submaximal currents), neurosteroids have been shown to in-

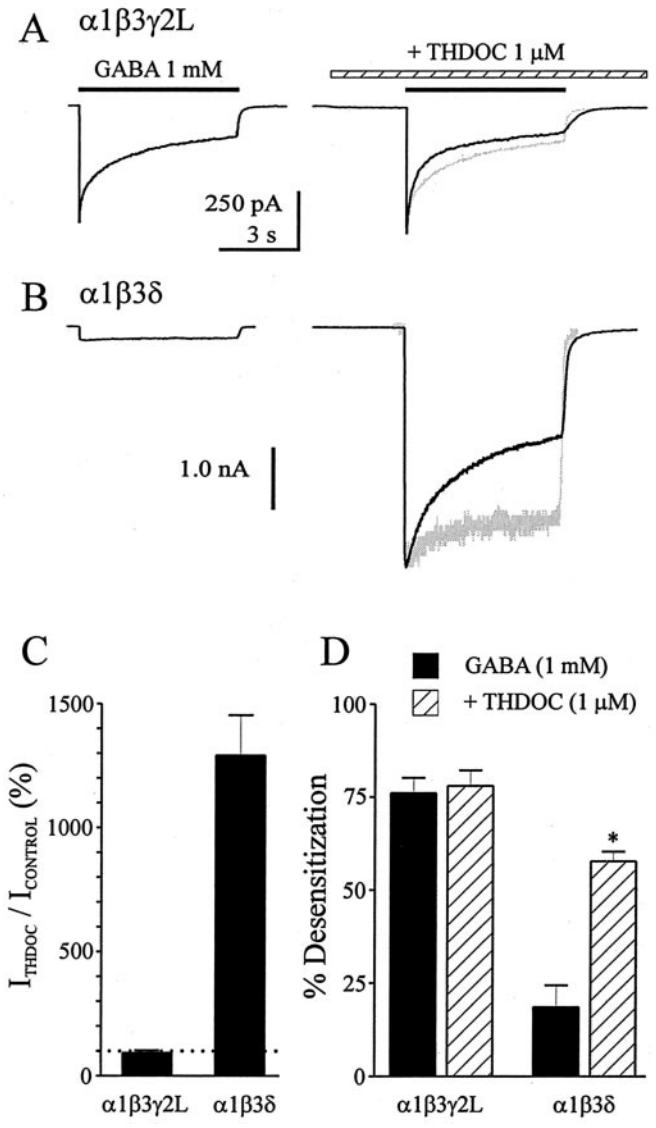

Figure 1. THDOC differentially modulated $\alpha1\beta3\gamma2L$ and $\alpha1\beta3\delta$ $GABA_A$ receptor currents. *A*, $\alpha1\beta3\gamma2L$ $GABA_A$ receptor currents evoked by GABA (1 mM; left trace) were minimally affected by pre-applied THDOC (1 μM; right trace). In this and subsequent figures, GABA applications are indicated by solid bars, and THDOC applications are indicated by hatched bars. *B*, $\alpha1\beta3\delta$ $GABA_A$ receptor currents evoked by GABA (1 mM; left trace) were markedly enhanced by pre-applied THDOC (1 μM; left trace). The time calibration is the same as in *A*. In *A* and *B*, the GABA alone trace was scaled to peak and overlaid (gray trace) for comparison of the time course. *C*, THDOC enhancement of peak current amplitude for $\alpha1\beta3\gamma2L$ and $\alpha1\beta3\delta$ $GABA_A$ receptors is summarized. The dotted line indicates 100%. *D*, The extent of desensitization measured with GABA alone (solid bars) and with THDOC (hatched bars) is compared. Data are mean ± SEM. $^*p < 0.05$, compared with GABA alone condition.

crease the gating efficacy of $GABA_A$ receptor single-channel currents in a manner that is unlikely to be explained simply by altered GABA binding (Mistry and Cottrell, 1990; Twyman et al., 1992). $\alpha1\beta3\gamma2L$ receptor single-channel currents are known to exhibit higher efficacy gating patterns (longer open times in long complex bursts) than $\alpha1\beta3\delta$ receptors [brief openings and brief bursts at both high and low GABA concentrations (Fisher and Macdonald, 1997)] in response to high concentrations of GABA (Fisher and Macdonald, 1997; Haas and Macdonald, 1999). Also, $\alpha1\beta3\gamma2L$ receptor single-channel currents show low-efficacy gating when low GABA concentrations are used (Fisher and Macdonald, 1997), and, under these conditions, THDOC enhancement is robust (Wohlfarth et al., 2002). If THDOC acts via stabilization of high-efficacy gating, and therefore selectively enhances $GABA_A$ receptors under conditions of low-efficacy gating, this might account not only for the GABA concentration-dependent modulation of $\alpha1\beta3\gamma2L$ receptors but also the apparent isoform preference of $\alpha1\beta3\delta$ over $\alpha1\beta3\gamma2L$ receptors. We tested this hypothesis in the following sections.

Increased THDOC enhancement of $\alpha1\beta3\gamma2L$ receptor currents evoked by a partial agonist

One complication of using low concentrations of GABA to favor a low-efficacy $\alpha1\beta3\gamma2L$ receptor gating is that modulators such as THDOC may alter GABA binding in addition to the gating pattern. We reasoned that a partial agonist (restricted to low-efficacy activation despite saturating concentration) would be a useful alternative to assess of the role of gating efficacy in $GABA_A$ receptor modulation by THDOC. The compound P4S has been characterized as a partial agonist acting at the GABA binding site. P4S evoked smaller amplitude currents than GABA on $\alpha\beta\gamma$ receptors, even at the saturating concentration of 1 mM (EC_{50} was similar to GABA) (Krogsgaard-Larsen et al., 1980, 1981; Ebert et al., 1997). Single-channel recording indicated previously that P4S evoked brief duration openings (Steinbach and Akk, 2001), similar to those observed from $\alpha1\beta3\gamma2L$ receptors with low concentrations of GABA and also to those observed with $\alpha1\beta3\delta$ receptor currents (even at high GABA concentration) (Fisher and Macdonald, 1997; Haas and Macdonald, 1999). Thus, P4S could be used to saturate the GABA binding site(s), yet induce only the low-efficacy gating pattern of $\alpha1\beta3\gamma2L$ receptors. Figure 2*A* shows the response of $\alpha1\beta3\gamma2L$ receptors to a saturating concentration of GABA or P4S from the same cell. Currents evoked by P4S (1 mM) were always smaller than currents evoked by GABA (1 mM) from the same cells (16.8 ± 2.8% of control; $n = 4$) and showed minimal extent of desensitization (19.7 ± 3.7%) (Fig. 2*D*). If gating efficacy was a critical factor for THDOC enhancement, then the low-efficacy gating favored by P4S (relative to 1 mM GABA) should be markedly increased by THDOC modulation of $\alpha1\beta3\gamma2L$ receptors, similar to the effects of modulators on $GABA_A$ receptor partial agonists shown previously (Maksay et al., 2000). Indeed, P4S-evoked $\alpha1\beta3\gamma2L$ receptor currents were strongly potentiated by pre-applied THDOC (598 ± 86%; $n = 4$) (Fig. 2*B*,*C*). This potentiation was accompanied by increased desensitization (Fig. 2*D*), similar to the effect of THDOC on $\alpha1\beta3\delta$ receptor currents (Fig. 1*D*), although the rate of desensitization was not as fast as that observed with GABA (1 mM) application to the same cell (Fig. 2*B*, inset; Table 1). The extent of desensitization during the 6 sec application of P4S in the presence of THDOC was similar to that observed for applications of GABA in the same cells (90.4 ± 2.1, compared with 82.3 ± 3.6%) (Fig. 2*D*). Interestingly, deactivation after removal of P4S was not significantly enhanced by THDOC (45.8 ± 13.2 msec; with THDOC, 58.0 ± 4.5 msec).

The current amplitudes recorded with GABA (1 mM) alone were not different from those recorded with P4S and THDOC together within individual cells (Fig. 2*C*). Identical experiments were performed on $\alpha1\beta3\delta$ receptors to determine whether P4S-evoked currents could be modulated to amplitudes greater than those observed with GABA alone. We confirmed that P4S is also a partial agonist at $\alpha1\beta3\delta$ receptors, because it evoked smaller currents than GABA (data not shown), similar to a recent report using $\alpha4\beta3\delta$ receptors (Brown et al., 2002). THDOC strongly enhanced P4S-evoked currents, which exceeded the amplitude of

Table 1. Effects of THDOC on desensitization and deactivation of $GABA_A$ receptor currents

	τ1	A1	τ2	A2	τ3	A3	%Des	Deact	*n*
γ2L	52.6 (14.5)	0.19 (0.05)	537 (123)	0.26 (0.04)	3236 (336)	0.37 (0.03)	76.1 (4.1)	210.8 (59.9)	5
+THDOC	81.8 (21.4)	0.22 (0.06)	436 (79.3)	0.32 (0.03)	3687 (749)	0.30 (0.02)	78.0 (4.2)	471.6* (26.4)	5
γ2L (P4S)							19.7 (3.7)	45.8 (13.2)	4
+THDOC			330.3 (52.6)	0.27* (0.02)	2168 (227)	0.40* (0.03)	90.4* (2.1)	58.0 (4.5)	4
δ (L9′S)							11.2 (9.4)	203.0 (19.6)	5
+THDOC			458.0 (65.0)	0.20 (0.03)	3693 (611)	0.41 0.03	50.3* (2.8)	1450* (52.0)	5
δ (L9′F)							12.9 (7.6)	77.4 (10.9)	5
+THDOC							19.1 (4.3)	226.6* (14.4)	5
δ					2016 (290)	0.33 (0.03)	18.6 (5.8)	92.1 (14.1)	8
+THDOC			594.8 (63.0)	0.28 (0.03)	3116 (490)	0.44 (0.04)	57.8* (2.6)	457.8* (94.2)	8

Data are presented for each isoform as mean (SEM). *Significant difference compared with values obtained with GABA alone; %Des, extent of desensitization; Deact, weighted time constant of deactivation.

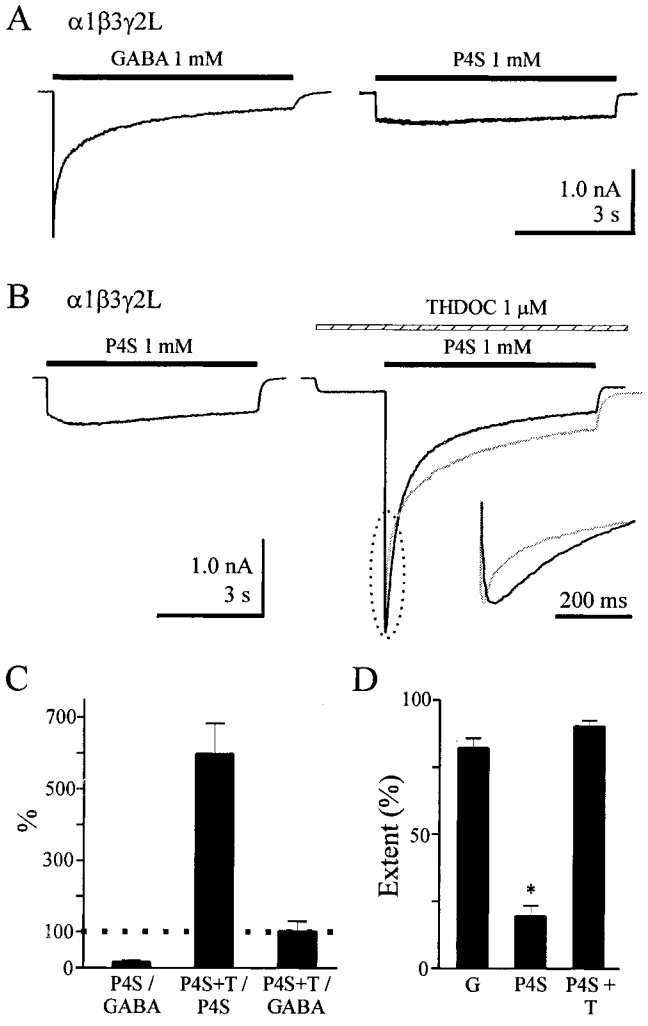

Figure 2. $\alpha1\beta3\gamma2L$ $GABA_A$ receptor currents evoked by the low-efficacy agonist P4S were enhanced by THDOC. *A*, Current traces recorded from the same cell expressing $\alpha1\beta3\gamma2L$ $GABA_A$ receptors in response to 1 mM GABA (left) or 1 mM P4S, a low-efficacy $GABA_A$ receptor agonist (right). *B*, $\alpha1\beta3\gamma2L$ receptor currents evoked by P4S (solid bar, left) were markedly enhanced by pre-applied THDOC (hatched bar, right). The response to GABA alone (1 mM) in the same cell is scaled and overlaid (gray) with the P4S plus THDOC current for comparison. The first 200 msec (indicated by the dotted oval) was expanded in the inset. *C*, Comparison of currents evoked by GABA, P4S, and P4S plus THDOC. For each cell, the P4S amplitude was <20% of the GABA-evoked current (left bar), the P4S plus THDOC amplitude was ~600% of the P4S amplitude (middle bar), and the P4S plus THDOC amplitude was not different from the GABA alone amplitude (right bar). The dotted line indicates 100%. *D*, The extent of desensitization is shown for currents evoked by GABA alone (G), P4S alone, and P4S plus THDOC (T). Asterisk indicates significant difference from GABA alone and GABA plus THDOC ($p < 0.0001$).

GABA-evoked (1 mM) currents (in the same cells) by ~10-fold (data not shown).

Gating efficacy, not desensitization, is a critical determinant of THDOC modulation of $GABA_A$ receptor currents

Despite the clear enhancement by THDOC of $\alpha1\beta3\gamma2L$ receptor currents evoked by P4S, we could not rule out the possibility that the minimal desensitization, not the low-gating efficacy, was the critical "permissive" factor involved in THDOC enhancement of $GABA_A$ receptor currents. In fact, desensitized states have been suggested to be important for THDOC modulation of $GABA_A$ receptors (Leidenheimer and Chapell, 1997; Zhu and Vicini, 1997). If $\alpha1\beta3\delta$ receptor currents (and P4S-evoked $\alpha1\beta3\gamma2L$ receptor currents) were selectively enhanced by THDOC because of their characteristically minimal desensitization, then one would predict that a high-efficacy, nondesensitizing, $GABA_A$ receptor isoform should also be markedly enhanced by THDOC. In an unrelated set of experiments, we found that a L9′S mutation in TM2 of the δ subunit clearly increased single-channel gating efficacy but did not alter macroscopic desensitization (Fig. 3). The currents evoked by a 400 msec application of 1 mM GABA to outside-out patches (to ensure resolution of possible fast phases) containing either $\alpha1\beta3\delta$ (Fig. 3*A*) or $\alpha1\beta3\delta$ (L9′S) receptors (Fig. 3*B*) demonstrated the minimal desensitization for both receptors. Single-channel analysis revealed longer duration openings that tended to occur in bursts (Fig. 3*C*), similar to the effects of L9′S–T mutations in $GABA_A$ receptor and nACh receptor channels (Filatov and White, 1995; Labarca et al., 1995; Bianchi and Macdonald, 2001). In contrast to $\alpha1\beta3\delta$ receptors that open to one of two brief-duration open states in response to 1 mM GABA (Fisher and Macdonald, 1997; Haas and Macdonald, 1999), the distribution of open durations for $\alpha1\beta3\delta$ (L9′S) receptors required three exponential functions with time constants of 0.54, 1.36, and 4.74 msec, with relative areas of 0.25, 0.57, and 0.18, respectively (Fig. 3*D*). The time constants and their fractional contributions were similar to our previous reports for $\alpha1\beta3\gamma2L$ receptors (Fisher and Macdonald, 1997; Haas and Macdonald, 1999), indicating that the mutation caused a clear shift toward higher efficacy gating.

Because $\alpha1\beta3\delta$ (L9′S) $GABA_A$ receptors showed high-efficacy gating (like $\alpha1\beta3\gamma2L$ receptors) but unaltered macroscopic desensitization (like $\alpha1\beta3\delta$ receptors), this construct provided an ideal tool for dissecting the potential roles of gating efficacy and desensitization in THDOC modulation. If THDOC enhancement was somehow limited by receptor desensitization (or an associated process), then the minimally desensitizing $\alpha1\beta3\delta$ (L9′S) receptors should still be robustly enhanced. However, if low-efficacy ($\alpha1\beta3\delta$-like) gating was a prerequisite for enhancement, then THDOC effects on peak current should be reduced by

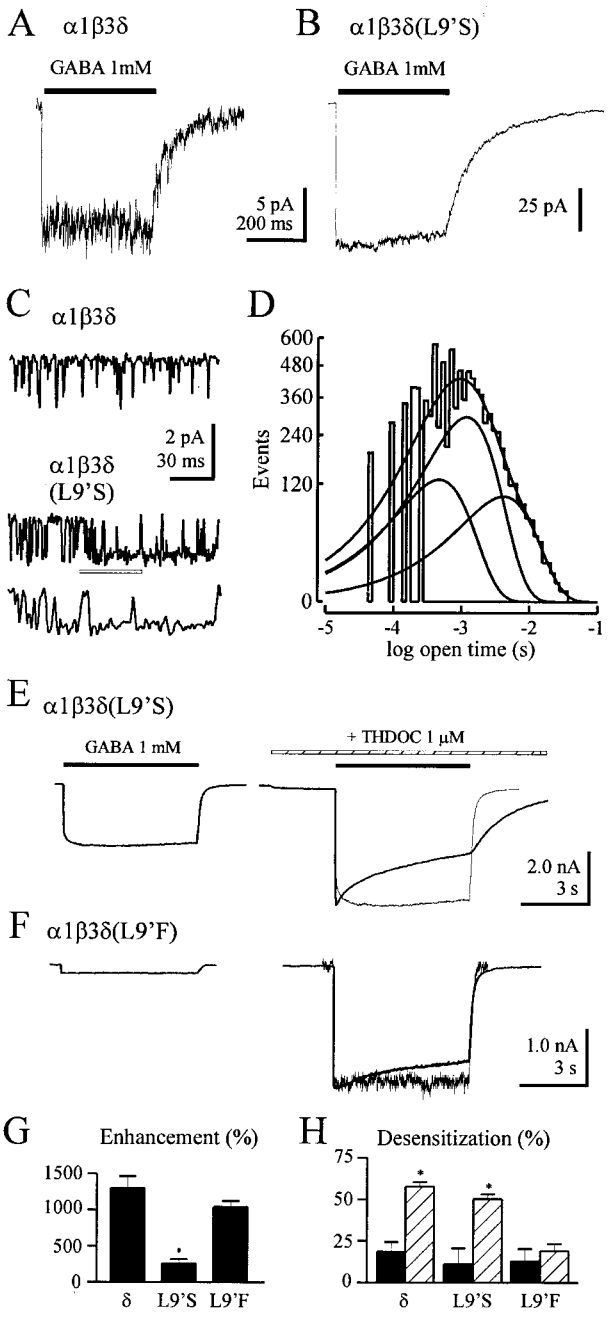

Figure 3. $\alpha1\beta3\delta$ (L9′S) $GABA_A$ receptors exhibited increased gating efficacy. *A*, *B*, Concentration–jump experiments (400 msec) performed on outside-out patches demonstrated minimal desensitization in response to GABA (1 mM) for $\alpha1\beta3\delta$ (*A*) and $\alpha1\beta3\delta$ (L9′S) (*B*) isoforms. *C*, Representative single-channel records are presented from $\alpha1\beta3\delta$ (top trace) and $\alpha1\beta3\delta$ (L9′S) receptors. A portion of the middle trace, indicated by the open bar, is expanded in the bottom trace. *D*, Event histogram of $\alpha1\beta3\delta$ (L9′S) receptor single-channel openings evoked by steady state application of GABA (1 mM). Data were pooled from two patches. The distribution was best described by the sum of three exponential functions. The individual fitted functions, as well as their sum, are shown as curves on the plot. See Results for time constants and relative areas. *E*, *F*, Currents evoked by GABA (1 mM; solid bar) applied to cells expressing $\alpha1\beta3\delta$ (L9′S) (*E*) and $\alpha1\beta3\delta$ (L9′F) (*F*) in the absence (left traces) and presence (right traces) of THDOC (1 μM; hatched bar). *G*, Extent of desensitization (percentage) for 6 sec applications of GABA in the absence (solid bars) or presence of pre-applied THDOC (1 μM; hatched bars).

the L9′S mutation because of its high efficacy ($\alpha1\beta3\gamma2$L-like) gating. We found that THDOC enhancement of $\alpha1\beta3\delta$ (L9′S) receptors was reduced more than fivefold compared with wild-type $\alpha1\beta3\delta$ receptors (262 ± 55%; $n = 5$) (Fig. 3*E*,*G*), consistent with the importance of low-efficacy gating (not minimal desensitization) for neurosteroid modulation. No additional enhancement was observed with increased THDOC concentration (3 μM; data not shown). Interestingly, despite the attenuated enhancement of peak current, the extent of desensitization was increased to 50.3 ± 2.8%, indistinguishable from the effect of THDOC on $\alpha1\beta3\delta$ receptor current desensitization (Fig. 3*H*). Because it was possible that the decreased THDOC enhancement was because of a structural requirement for the conserved 9′ leucine (and not because of the higher efficacy gating of the L9′S mutation), we studied an additional mutation at that site, $\alpha1\beta3\delta$ (L9′F). THDOC enhancement of this isoform was robust and indistinguishable from enhancement of $\alpha1\beta3\delta$ receptors (Fig. 3*F*,*G*) (1043 ± 81%; $n = 5$). Although we have not investigated the single-channel gating characteristics of receptor channels with the δ (L9′F) mutation, the unaltered deactivation rate argued against an increase in gating efficacy for this mutant (Table 1). In any case, the clear enhancement of $\alpha1\beta3\delta$ (L9′F) receptor currents by THDOC excluded a nonspecific requirement for the 9′ leucine as an explanation for the $\alpha1\beta3\delta$ (L9′S) results. Desensitization of $\alpha1\beta3\delta$ (L9′F) receptor currents was not significantly increased in the presence of THDOC (Fig. 3*H*) (12.9 ± 7.5% with GABA alone; 19.1 ± 4.3% with THDOC), in contrast to the effect of THDOC on $\alpha1\beta3\delta$ and $\alpha1\beta3\delta$ (L9′S) receptor currents. We also used P4S (1 mM) to evoke currents from the high-efficacy $\alpha1\beta3\delta$ (L9′S) receptors and observed partial agonism with maximal currents that were ~50% smaller than those evoked by GABA (1 mM) for that isoform. Accordingly, THDOC (1 μM) enhancement of P4S-evoked currents using $\alpha1\beta3\delta$ (L9′S) receptors was approximately twice the enhancement observed with currents evoked by 1 mM GABA (data not shown).

Decreased neurosteroid enhancement of $\alpha1\beta3\delta$ receptor currents evoked by β-alanine

Despite the clear reduction in THDOC enhancement of $\alpha1\beta3\delta$ (L9′S) receptor currents, we endeavored to alter the gating efficacy of $\alpha1\beta3\delta$ receptors without introducing any mutations. Two recent studies (Adkins et al., 2001; Brown et al., 2002) demonstrated that the synthetic GABA analog tetrahydroisoxazolopyridinol (THIP) evoked maximal responses that were ~70% larger than those evoked by saturating GABA for $\alpha4\beta3\delta$ receptors (but not $\alpha4\beta3\gamma2$ receptors), suggesting that GABA might not be a full agonist at $\alpha\beta\delta$ receptors. Although we observed similar results with THIP on $\alpha1\beta3\delta$ receptors (data not shown), we found even greater "superagonism" using the endogenous amino acid β-alanine. β-alanine did not show increased efficacy compared with GABA at $\alpha1\beta3\gamma2$L receptors (data not shown). The β-alanine concentration–response relationship for $\alpha1\beta3\delta$ receptors is shown in Figure 4*A*, normalized to the maximum GABA response (evoked by 1 mM GABA) in each cell. Clearly a higher efficacy $\alpha1\beta3\delta$ receptor gating pattern was accessible with β-alanine than with GABA, because high concentrations (200 mM) of β-alanine evoked currents that were over 600% of the maximal GABA-evoked current amplitude ($n = 3$). Although these concentrations were much higher than are likely to occur in the brain, β-alanine provided an additional tool to test the hypothesis that THDOC preferentially enhanced $GABA_A$ receptor currents under conditions that favored low-efficacy gating. First, we confirmed that THDOC robustly enhanced $\alpha1\beta3\delta$ receptor currents evoked by low-concentration β-alanine (presumed to be low-efficacy, on the basis of similar macroscopic current amplitude and desensitization to currents evoked by 1 mM GABA). Indeed, currents evoked by 2 mM β-alanine were enhanced

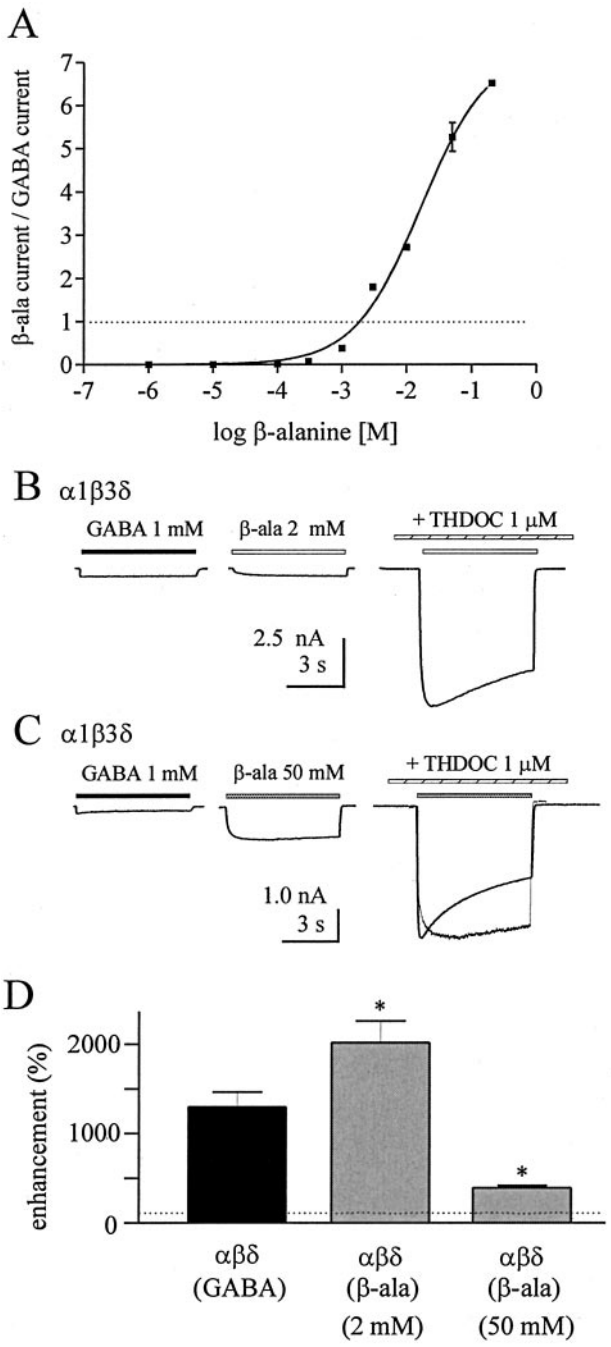

Figure 4. Attenuated neurosteroid enhancement of $\alpha1\beta3\delta$ receptor currents evoked by high, but not low, concentrations of β-alanine. *A*, Concentration–response relationship for $\alpha1\beta00$ receptor currents evoked by β-alanine. Currents were normalized to the amplitude of a maximal GABA response evoked in each cell by 1 mM GABA (dotted line). Data were from three cells. *B*, Currents evoked by GABA (1 mM; left trace) and β-alanine (2 mM; middle trace) from the same cell, as well as the enhancement of β-alanine current by pre-applied THDOC (1 μM; right trace). *C*, Same protocol as in *B*, except that 50 mM β-alanine was used, a concentration that evoked currents approximately fivefold larger than those evoked by GABA. *D*, Summary of THDOC enhancement of peak current under various conditions. Asterisk indicates significant difference from THDOC enhancement of currents evoked by GABA (1 mM).

~2000% by THDOC (Fig. 4*B*). Our prediction was that currents evoked by a higher concentration of β-alanine (a condition of high-efficacy gating, on the basis of peak currents, relative to currents evoked by GABA) would be less sensitive to THDOC modulation. THDOC enhancement of $\alpha1\beta3\delta$ currents evoked by 50 mM β-alanine was significantly decreased (393.5 ± 21.8%; n = 4), consistent with attenuated THDOC modulation of high-efficacy $GABA_A$ receptor currents. The extent of desensitization was increased from 14.5 ± 3.8 to 59.0 ± 8.0% by THDOC, similar to the effect of THDOC on GABA-evoked currents.

Are allosteric shifts in $\alpha1\beta3\delta$ receptor gating efficacy limited to neurosteroids?

To determine whether gating efficacy might be a general target for $GABA_A$ receptor modulation, we investigated the effects of an additional compound, mefenamic acid, a nonsteroidal anti-inflammatory drug known to modulate $GABA_A$ receptor currents (Halliwell et al., 1999). Mefenamic acid (MFA) (30 μM) enhanced maximal GABA-evoked $\alpha1\beta3\delta$ receptor currents by more than 1000%, similar to the effects of THDOC. In contrast, pre-applied MFA had little effect on maximal GABA-evoked currents for $\alpha1\beta3\gamma2L$ receptors. Although these results were consistent with MFA targeting reluctant $GABA_A$ receptors, additional evidence for modulation of gating efficacy would be to restore MFA enhancement of $\alpha1\beta3\gamma2L$ receptor currents under a condition of low-gating efficacy. Therefore, we evoked $\alpha1\beta3\gamma2L$ receptor currents with a low concentration of GABA, known to elicit small macroscopic currents and primarily brief-duration (low-efficacy), single-channel openings (Fisher and Macdonald, 1997). MFA markedly enhanced these currents (Fig. 5*C*), consistent with the idea that shifts in gating efficacy may be a general mechanism for regulation of $GABA_A$ receptor function.

THDOC enhancement of $GABA_A$ receptor currents evoked by the endogenous partial agonist taurine

Taurine is an endogenous amino acid that has partial agonist activity at $GABA_A$ receptors in addition to agonist activity at glycine receptors. Its basal extracellular concentration is thought to be ~10–20 μM (Lerma et al., 1986). We investigated whether THDOC could also enhance the small currents evoked by taurine for $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2L$ receptors. $\alpha1\beta3\gamma2L$ receptor currents evoked by a saturating concentration of taurine (20 mM) were markedly enhanced by THDOC (515 ± 104%; n = 4) (Fig. 6*A*,*D*). A maximal current evoked by GABA (1 mM) from the same cell is shown for comparison. The same protocol was applied to $\alpha1\beta3\delta$ receptor currents, and they were markedly enhanced (2030 ± 440%; n = 4) (Fig. 6*B*,*D*). In contrast to $\alpha1\beta3\gamma2L$ receptors, the maximal THDOC-modulated $\alpha1\beta3\delta$ receptor currents were clearly larger than the maximal GABA-evoked current in the same cells. Finally, we used lower concentrations of taurine (10 μM) and THDOC (100 nM) to more closely approximate physiological conditions under which THDOC modulation of $GABA_A$ receptors might occur (Paul and Purdy, 1992). Experiments were performed on high-affinity $\alpha6\beta3\delta$ receptors, known to be expressed exclusively in extrasynaptic membrane in the cerebellum (Nusser et al., 1998), where they contribute to tonic forms of cerebellar inhibition (Rossi and Hamann, 1998; Hamann et al., 2002). Taurine evoked small amplitude currents from $\alpha6\beta3\delta$ receptors (<20 pA) (Fig. 6*C*, left trace, *D*). In the presence of THDOC, taurine current amplitudes were enhanced 449 ± 36% (n = 4) (Fig. 6*C*, middle trace). The direct agonist action of 100 nM THDOC could be observed during the pre-application, consistent with our previous study indicating a high apparent affinity of this isoform for THDOC (Wohlfarth et al., 2002). For comparison, the response to 1 μM GABA was shown in the same cell to indicate that the currents evoked by taurine, even modulated by THDOC, were small compared with GABA-evoked currents.

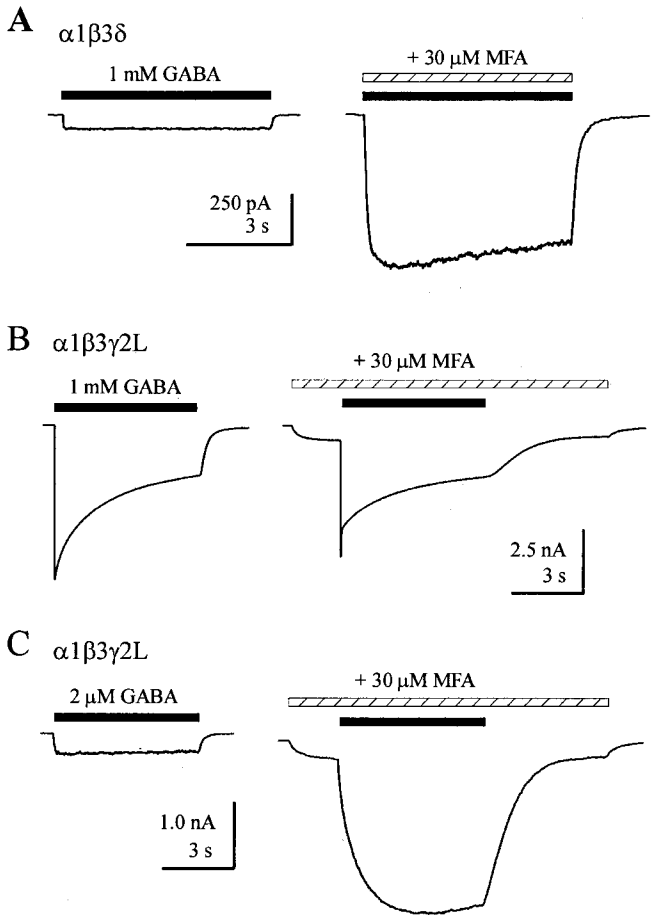

Figure 5. The nonsteroidal ibuprofen analog MFA differentially modulated $\alpha1\beta3$d and $\alpha1\beta3\gamma2$L $GABA_A$ receptor currents. *A*, $\alpha1\beta3\delta$ receptor currents evoked by 1 mM GABA alone (left trace, solid bar) were markedly enhanced by co-applied 30 μM MFA (hatched bar, right trace). *B*, $\alpha1\beta3\gamma2$L receptor currents evoked by 1 mM GABA alone (left) or with pre-applied 30 μM MFA (right). *C*, $\alpha1\beta3\gamma2$L receptor currents evoked by 2 μM GABA were potentiated by 30 μM MFA. Similar results were obtained in at least three additional cells for each condition.

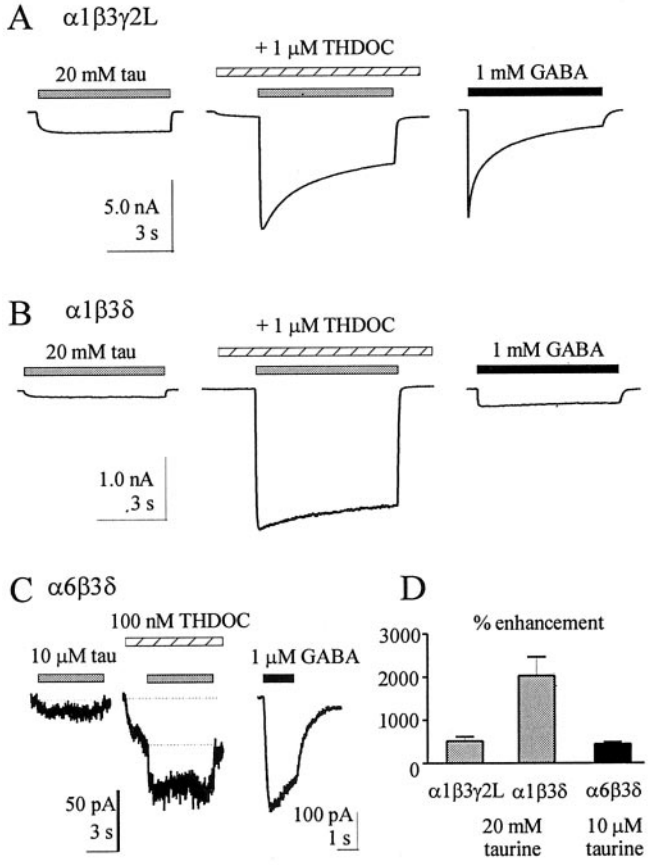

Figure 6. THDOC enhancement of $GABA_A$ receptor currents evoked by the endogenous partial agonist taurine. *A*, $\alpha1\beta3\gamma2$L receptor current evoked by 20 mM taurine alone (solid bar, left trace) or with pre-applied 1 μM THDOC (hatched bar, middle trace). The maximal GABA-evoked current is shown in the same cell for comparison (1 mM; solid bar, right trace). Scale bars apply to all three traces, and the time bar applies to other panels (except *C*, right trace). *B*, $\alpha1\beta3\delta$ receptor current evoked by 20 mM taurine alone (solid bar, left trace) or with pre-applied 1 μM THDOC (hatched bar, middle trace). The maximal current evoked by 1 mM GABA is shown from the same cell (solid bar, right trace). *C*, $\alpha6\beta3\delta$ receptor current evoked by 10 μM taurine alone (solid bar, left trace) or with pre-applied 100 nM THDOC (hatched bar, middle trace). The response to 1 μM GABA from the same cell is shown (right trace; note the different horizontal and vertical scale bars). The dotted line (left trace) is a baseline reference for the small taurine-evoked current. *D*, Summary plot indicating peak current enhancement by THDOC for the various conditions ($n = 4$ cells for each).

It is worth noting that Hamann et al. (2002) suggested that $\alpha6\beta$xδ receptors that appear to mediate tonic inhibition in the cerebellum are insensitive to neurosteroids, consistent with initial studies by Zhu et al. (1996) but in contrast to this and other studies (Adkins et al., 2001; Brown et al., 2002; Wohlfarth et al., 2002). One possible explanation for the results of Hamann et al. (2002) is the use of prolonged applications of THDOC (10–30 sec) in their neuronal preparation. We have shown that THDOC causes rapid and nearly complete desensitization of $\alpha6\beta3\delta$ receptor currents within 6 sec (using 1 mM GABA and 1 μM THDOC), raising the possibility that extensive desensitization developed within the time course of their delivery of THDOC, off-setting potential enhancement. However, we cannot rule out the possibility that endogenous δ subunit-containing receptors behave differently than those expressed in recombinant systems (Cooper et al., 1999; Fancsik et al., 2000), possibly attributable to phosphorylation state or interactions with other membrane or cytoplasmic proteins.

Discussion

Although many pharmacological agents exhibit subunit specificity for $GABA_A$ receptors (Macdonald and Olsen, 1994; Mehta and Ticku, 1999), the basis for this selectivity may, in some instances, be more complex than the presence or absence of the binding site(s). The neurosteroid THDOC caused a marked increase in the maximal GABA-evoked currents of $\alpha1\beta3\delta$ receptors, accompanied by a shift in gating toward the high-efficacy bursting pattern observed with $\alpha1\beta3\gamma2$L receptors (Wohlfarth et al., 2002). In contrast, $\alpha1\beta3\gamma2$L receptor peak currents were not significantly modulated by THDOC when currents were evoked by saturating GABA but were enhanced when evoked by low GABA concentrations. This prompted us to investigate whether the observed selectivity of THDOC modulation depended on subunit composition or the distinct functional properties of each isoform. We exploited differences in gating efficacy that were dependent not only on receptor subunit composition ($\alpha\beta\delta$ vs $\alpha\beta\gamma$) but also on the type of agonist (partial vs full) used to activate the receptors. Our results suggested that THDOC (and perhaps other modulators) acted by producing a shift in channel activity from low-efficacy to high-efficacy gating patterns. The observations (from this and previous studies) that $GABA_A$ receptor efficacy is a function not only of subunit composition but also of the con-

centration and identity of the agonist used suggested that THDOC modulation can effectively "distinguish" receptor populations on the basis of subunit composition as well as on functional behavior within a given receptor population.

THDOC modulation: targeting $GABA_A$ receptor-gating activity independent of subunit composition

Much attention has focused on generation and characterization of subunit-specific $GABA_A$ receptor modulators. Allosteric modulation, on the basis of functional differences (such as gating efficacy or desensitization) rather than differences in primary structure per se, may represent an alternative basis for isoform-specific modulation. Although subunit composition can influence functional properties, channel behavior is also dependent on agonist identity, concentration, and allosteric modulation. Thus, $GABA_A$ receptor function is not uniquely specified by subunit composition, and targeting receptors on the basis of gating efficacy may represent a novel cross-section of isoforms. Also, neurosteroid modulation has the potential to regulate a given isoform differently, depending on its level of activation. The observations that both endogenous (THDOC) and exogenous (MFA) modulators are capable of scaling the efficacy of $\alpha\beta\delta$ $GABA_A$ receptor function has important implications for the *in vivo* regulation of tonic inhibition as well as possible therapeutic targeting of this inhibition. Tonic forms of inhibition are likely to be mediated by low concentrations of GABA or other GABA-mimetics present in the extracellular space (Lerma et al., 1986). Because $\alpha\beta\delta$ isoforms are thought to mediate tonic inhibition by sensing extrasynaptic neurotransmitter (Nusser et al., 1998), their low-efficacy gating may allow for a dynamic regulation of neuronal excitability through allosteric modulators. Increasing the concentration of GABA or taurine may be a relatively inefficient mechanism for increasing tonic inhibitory drive, in part because these agonists fail to fully activate $\alpha\beta\delta$ isoforms even at very high concentrations. Shifting the gating efficacy of $\alpha\beta\delta$ receptors through the release of endogenous modulators like THDOC may present an alternative mechanism for regulating inhibition.

Dissociating effects of THDOC on gating efficacy and desensitization

Whether the described correlation between gating efficacy and desensitization for $\alpha1\beta3\delta$ and $\alpha1\beta3\gamma2L$ receptors was coincidental or represented a coupling of these processes was unknown. THDOC increased both $\alpha1\beta3\delta$ receptor macroscopic desensitization and single-channel gating efficacy, consistent with the coupling of these processes (Wohlfarth et al., 2002). Also, THDOC increased the maximal amplitude and desensitization of P4S-evoked currents from $\alpha1\beta3\gamma2L$ receptors, consistent with this hypothesis. However, several other observations from this study were consistent with a dissociation of these processes. The δ (L9'S) mutation clearly increased gating efficacy without altering desensitization, suggesting that the two processes could be dissociated. Also, THDOC could alter peak amplitude and desensitization independently. Desensitization was increased despite relatively small enhancement of peak current for the δ (L9'S) mutation, whereas minimal desensitization was observed despite more than 10-fold increases in peak amplitude for the δ (L9′F) mutant and taurine-evoked currents from wild-type $\alpha1\beta3\delta$ receptors. Evaluation of an isoform exhibiting fast desensitization but low-gating efficacy would support this proposed dissociation. Despite this evidence for independent modulation of gating and desensitization, additional work on this issue is necessary because it is apparent that macroscopic changes in desensitization may not necessarily reflect altered desensitized states per se (Bianchi and Macdonald, 2001).

Gating efficacy as a general target for allosteric modulation of ion channels

$GABA_A$ receptor gating efficacy appears to be specified by a combination of subunit composition, agonist identity, agonist concentration, and allosteric modulation. Allosteric control of gating efficacy is observed in many systems, suggesting a general mechanism for tuning channel-mediated electrical signaling. The more generalized phenomenon of modal gating has been described in several types of ligand- and voltage-gated channels, and gating patterns can be altered by subunit composition (Naranjo and Brehm, 1993; Fisher and Macdonald, 1997), mutation (Milone et al., 1998; Wang et al., 2000; Zhong et al., 2001), phosphorylation (Yue et al., 1990; Marrion, 1996), G-protein interaction (Delcour and Tsien, 1993), allosteric modulators (Hess et al., 1984; Twyman et al., 1989; Twyman and Macdonald, 1992; Wohlfarth et al., 2002), and other factors (Zhou et al., 1991; Marrion, 1993; Herlitze et al., 2001; Schonherr et al., 2002).

Allosteric modulation sets the gain of agonist–receptor interactions

It is often assumed that GABA is a full agonist at all $GABA_A$ receptor isoforms. Determination of full agonism is a relative one, requiring a comparison with other known agonists. The possibility that GABA is a partial agonist at $\alpha\beta\delta$ isoforms was specifically suggested by the observation that THIP (Adkins et al., 2001; Brown et al., 2002) and β-alanine (this study) evoked currents that were larger than those evoked by GABA alone. The increased modulation $\alpha\beta\delta$ receptors can be explained most simply if GABA is acting as a partial agonist, with larger observed enhancement attributable to the limited baseline level of activation. Consistent with this idea, the volatile anesthetic isofluorane and the nonbenzodiazepine anxiolytic tracazolate were shown to enhance maximal GABA-evoked currents of $\alpha1\beta1\delta$ but not $\alpha1\beta1\gamma2$ receptors (Lees and Edwards, 1998; Thompson et al., 2002). Enhancing the activity of partial agonists with allosteric modulators has been reported for $GABA_A$ and ATP-gated receptor channels (Kristiansen and Lambert, 1996; Khakh et al., 1999; Maksay et al., 2000; O'Shea et al., 2000).

Finally, the results suggest a potential mechanism for regulating extrasynaptic $GABA_A$ receptor currents evoked by partial agonists present in the extracellular space. The role of taurine in $GABA_A$ receptor function has remained elusive in part because of its weak agonism. If endogenous modulators such as neurosteroids augmented the response of native $GABA_A$ receptors to partial agonists, they may serve to reversibly regulate the gain of tonic inhibition. It is intriguing to consider the extreme case, in which a very weak partial agonist acted as a competitive antagonist capable of reversibly converting to full agonism by THDOC or other modulators. The role of tonic inhibition for CNS function has been the focus of several recent studies (Brickley et al., 1996, 2001; Bai et al., 2001; Hamann et al., 2002; Stell and Mody, 2002; Wu et al., 2003), but additional work is necessary to clarify the specific isoforms responsible, the physiological and pathophysiological relevance of this inhibition, and the roles of partial agonists and allosteric modulators in the regulation of tonic inhibition.

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