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# The Contraceptive Agent Provera Enhances $GABA_A$ Receptor-Mediated Inhibitory Neurotransmission in the Rat Hippocampus: Evidence for Endogenous Neurosteroids?

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**Neurosteroids typified by 5α-pregnan-3α-ol-20-one (5α3α) have emerged as the most potent endogenous positive modulators of the $GABA_A$ receptor, the principal mediator of fast inhibitory transmission within the CNS. Neurosteroids can be synthesized *de novo* in the brain in levels sufficient to modulate $GABA_A$ receptor function and, thus, might play an important physiological–pathophysiological role. Indirect support for this proposal comes from the observation that neurosteroid action is region and neuron selective. However, the mechanism(s) that imparts specificity of action remains primarily elusive. Although neurosteroids are relatively promiscuous toward different $GABA_A$ receptor isoforms, the contribution of local neurosteroid metabolism has been relatively unexplored. Here, we investigate the role of neurosteroid metabolism by using electrophysiological techniques to compare the actions of 5α3α and its metabolically stable synthetic analog ganaxolone on inhibitory neurotransmission in CA1 and dentate gyrus neurons. Furthermore, we evaluate the contribution of a key enzyme in neurosteroid metabolism [i.e., 3α-hydroxysteroidoxidoreductase (3α-HSOR)] to the inactivation of endogenous, or exogenously applied 5α3α. We show that low concentrations of ganaxolone, but not of 5α3α, enhance inhibitory transmission in dentate gyrus, whereas both steroids are similarly effective in CA1 neurons. Furthermore, inhibition of 3α-HSOR by the contraceptive agent Provera results in enhanced synaptic and extrasynaptic $GABA_A$ receptor-mediated inhibition in the dentate gyrus but not in the CA1 region. Collectively, these findings advocate a crucial role for local steroid metabolism in shaping $GABA_A$ receptor-mediated inhibition in a regionally dependent manner and suggest a novel action by the contraceptive agent on inhibitory centers in the CNS.**

*Key words:* **neurosteroid; inhibitory synaptic transmission; $GABA_A$ receptor; tonic current; hippocampus; patch-clamp**

## Introduction

A number of independent investigations have established that certain endogenous neurosteroids can potently and selectively enhance the action of the inhibitory neurotransmitter GABA at the $GABA_A$ receptor (Paul and Purdy, 1992; Lambert et al., 2001; Smith, 2002). Their behavioral repertoire is consistent with an enhancement of inhibition (i.e., they display anxiolytic, anticonvulsant, and analgesic activity) and, at higher doses, they are hypnotic and anesthetic (Belelli et al., 1990; Lambert et al., 1995). Neuroactive steroids might also be involved in the physiological and pathophysiological regulation of neuronal inhibition. Physiological levels of the most potent neurosteroid [i.e., 5α-pregnan-3α-ol-20-one (5α3α)] fall within the range of concentrations (≥10 nM) shown *in vitro* to enhance $GABA_A$ receptor function (Belelli et al., 1996a,b, 2002). Furthermore, neurosteroids can be synthesized *de novo* in the brain independent of peripheral sources both in glia and neurons. Thus, 5α3α can be synthesized from progesterone via the sequential action of 5α-reductase and 3α-hydroxysteroidoxidoreductase (3α-HSOR). Interestingly, in contrast to 5α-reductase, 3α-HSOR is involved in both the synthesis and degradation of 5α3α because, depending on whether the cytosolic or membrane-bound isoform prevails, it can either reduce 5α-dihydroprogesterone (5α-DHP) to 5α3α or oxidize 5α3α back to 5α-DHP, respectively (Mellon and Vaudry, 2001). The fact that the brain expression pattern and activity of the enzymes responsible for neurosteroid synthesis and degradation will selectively impact on $GABA_A$ receptor-mediated inhibition is supported by the following observations: (1) the activity of the enzymes involved in neurosteroid metabolism exhibits regional variations (Mellon and Vaudry, 2001), (2) administration of finasteride, an inhibitor of 5α-reductase, causes a dramatic decrease in rat brain 5α3α levels and concomitant changes in behavior consistent with the loss of an inhibitory tone (Matsumoto et al., 1999; Pinna et al., 2000), and (3) in animal models of psychiatric illness, inhibitory dysfunction appears to be associated with a loss of 5α-reductase activity (Dong et al., 2001). Findings from this and other laboratories have suggested that neurosteroid modulation of inhibitory transmission is neuron specific, a heterogeneity that might be conferred in part by the isoform of the $GABA_A$

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receptor and post-translational modifications of the receptor, including phosphorylation (Cooper et al., 1999; Belelli et al., 2002; Harney et al., 2003; Koksma et al., 2003). However, the contribution of local neurosteroid metabolism to neurosteroid action on $GABA_A$ receptor-mediated neurotransmission has received little attention. Thus, we compared the actions of 5α3α and its metabolically stable synthetic analog ganaxolone (3β-methyl-3α-ol-5α-pregnan-20-one) in hippocampal CA1 pyramidal neurons and dentate gyrus (DG) granule cells. Additionally, we evaluated the contribution of 3α-HSOR activity to the inactivation of endogenous or exogenously applied 5α3α by using two structurally distinct inhibitors of 3α-HSOR, the anti-inflammatory agent indomethacin, and the commonly used contraceptive progestin, Provera. Interestingly, Provera is also effective in the treatment of catamenial epilepsy (Newmark and Penry, 1980). The results presented here advocate a crucial role for local steroid metabolism in shaping $GABA_A$ receptor-mediated inhibition in a regionally dependent manner. Furthermore, the findings with Provera imply that the contraceptive agent might alter the function of inhibitory centers in the CNS.

## Materials and Methods

*Recordings from rat hippocampal slices*

*Slice preparation.* Hippocampal slices were prepared from Sprague Dawley rats of either sex [postnatal day (P) 16–P24] according to standard protocols described previously (Reynolds et al., 2003). Animals were killed by cervical dislocation in accordance with Schedule 1 of the United Kingdom Government Animals (Scientific Procedures) Act, 1986. The brain was rapidly removed and placed in oxygenated ice-cold artificial CSF (aCSF) solution containing the following (in mM): 225 sucrose, 2.95 KCl, 1.25 $NaH_2PO_4$, 26 $NaHCO_3$, 0.5 $CaCl_2$, 10 $MgSO_4$, 10 Glucose, 1 ascorbic acid, and 3 pyruvic acid bubbled with 95% $O_2$/5% $CO_2$ to give a pH of 7.4 (330–340 mOsm). The tissue was maintained in ice-cold aCSF while 300 μm horizontal slices were cut using a Vibratome (Intracel, Royston, UK). The slices were placed in an incubation chamber, consisting of a beaker and a nylon mesh-covered plastic ring on which the slices lay, filled with circulating, oxygenated, extracellular solution containing the following (in mM): 126 NaCl, 2.95 KCl, 26 $NaHCO_3$, 1.25 $NaH_2PO_4$, 2 $CaCl_2$, 10 D-glucose, and 2 $MgCl_2$, pH 7.4, when bubbled with 95% $O_2$/5% $CO_2$ (300–310 mOsm). Slices were incubated at 32°C for 1 hr and subsequently allowed to cool at room temperature before being used for recordings.

*Electrophysiology.* Whole-cell patch-clamp recordings were made at 35°C from hippocampal CA1 pyramidal neurons and dentate granule cells visually identified with a Zeiss 2FS (Zeiss, Welwyn Garden City, UK) or Olympus BX51 (Olympus, Southall, UK) microscope equipped with differential interference contrast-infrared optics. Patch pipettes were prepared from thick-walled borosilicate glass (Garner Glass, Claremont, CA) and had open tip resistances of 3–5 MΩ when filled with an intracellular solution that contained the following (in mM): 140 CsCl, 10 HEPES, 10 EGTA, 2 Mg-ATP, 1 $CaCl_2$, 5 QX-314, pH 7.3, with CsOH, 295–305 mOsm. Miniature IPSCs (mIPSCs) were recorded using an Axopatch 1D or Axopatch 200B amplifier (Axon Instruments, Foster City, CA) at a holding potential of −60 mV in an extracellular recording solution (see above) also containing 2 mM kynurenic acid (Sigma, Poole, UK) and 0.5 μM tetrodotoxin (TTX; Tocris, Bristol, UK) to block ionotropic glutamate receptors and action potentials, respectively. To ensure a reliable detection of the tonic current in dentate granule cells, experiments investigating the influence of steroid or Provera were performed in the absence of TTX on slices previously incubated in the presence of the GABA transaminase inhibitor, 50 μM vigabatrin (Sigma) for 2–4 hr (Yeung et al., 2003). Series resistance ranged from 6–18 MΩ and was compensated up to 80%. In each case, currents were sampled at 10 kHz and filtered at 2 kHz using an 8-pole low-pass Bessel filter.

*Drug application.* 5α3α, ganaxolone, indomethacin, and Provera were prepared as concentrated (1000×) stock solutions in DMSO while pentobarbital and bicuculline methobromide ($10^{-2}$ M) were dissolved in water. These stock solutions were diluted in the extracellular solution to the desired concentration. The final maximum DMSO concentration (0.2%) had no effect on any of the mIPSC parameters or the tonic current. Preliminary time course experiments revealed that the steroid and 3α-HSOR inhibitors effect was maximum after 5–8 min of exposure and stable afterward up to 30 min. Thus, all modulatory agents were applied via the perfusion system (2–4 ml/min) and allowed to infiltrate the slice for a minimum of 10 min before recordings were acquired. With the exception of 5α3α and ganaxolone, which were generous gifts from Dr. K. Gee (University of California, Irvine, CA), all drugs tested were obtained from Sigma.

*Data analysis.* Data were recorded onto a digital audiotape (DAT) using a Bio-Logic (Claix, France) DTR 1200 recorder and analyzed offline using the Strathclyde Electrophysiology Software, WinEDR/WinWCP (courtesy of Dr. J. Dempster, University of Strathclyde, Glasgow, UK). Individual mIPSCs were detected using a −4 pA amplitude threshold detection algorithm and visually inspected for validity. Accepted events were analyzed with respect to peak amplitude, 10–90% rise time, charge transfer, and time for events to decay by 50% (T50) and 90% (T90). To minimize the contribution of dendritically generated currents, which are subject to the unquantifiable effects of cable filtering, analysis was limited to those events that fell within the limits of a Gaussian distribution that describes the peak of the rise-time histogram. Analysis of rise-time histograms generally revealed a skewed distribution with a clear peak below 1 msec. Miniature IPSCs were fitted with either one or two exponentials given by the equations: $y(t) = A \cdot e^{(-t/\tau)}$, for mono-exponential mIPSCs; and $y(t) = A_1 \cdot e^{(-t/\tau 1)} + A_2 \cdot e^{(-t/\tau 2)}$ for bi-exponential mIPSCs, where $A$ is amplitude, $t$ is time, and $\tau$ is the decay time constant. An improvement of the fit by two exponentials compared with one resulted in a reduction in the SD of the residuals and was confirmed by use of the F-test. To compare mono-exponentially versus bi-exponentially decaying events recorded from each cell, a weighted decay time constant ($\tau_w$) was also calculated for those mIPSCs exhibiting a bi-exponential decay ($\tau_w = \tau_1 \cdot P_1 + \tau_2 \cdot P_2$, where $\tau_1$ and $\tau_2$ are the decay time constants of the first and second exponential functions, and $P_1$ and $P_2$ are the proportions of the current amplitude described by each component.). Individual $\tau$ values derived from mono-exponentially decaying events and $\tau_w$ values were averaged together to give a cumulative $\tau$ value. Tonic current amplitude was calculated as the difference between the holding current before and after application of 30 μM bicuculline methobromide (Brickley et al., 1996).

All results are reported as the arithmetic mean ± SEM. The large sample approximation of the Kolmogorov–Smirnoff (KS) test (SPSS software; SPSS, Chicago, IL) was used to compare the distribution of the mIPSCs parameters. Statistical significance of mean data was assessed with the unpaired Student's *t* test or repeated measures ANOVA *post hoc* followed by the Newman–Keuls test as appropriate, using the SigmaStat (SPSS) software package.

*Recordings from* Xenopus laevis *oocytes. Xenopus laevis* oocytes (stage V-VI) were isolated as described previously (Belelli et al., 1996a) and injected into their cytoplasm with 20–40 nl of cRNA transcripts (1 mg/ml) prepared from linearized human $GABA_A$ $\alpha_1$, $\beta_3$, and $\gamma_2$ cDNA according to standard protocols. Two to twelve days after injection, recordings were performed on such oocytes voltage-clamped at −60 mV with an Axoclamp 2A (Axon Instruments) in the twin-electrode voltage-clamp mode. The voltage and current-passing electrodes were filled with 3 M KCl and had resistances of 0.8–2 MΩ when measured in the recording solution (frog Ringer). The oocytes were continually superfused with frog Ringer containing the following (in mM): 120 NaCl, 1.8 $CaCl_2$, 2 KCl, and 5 HEPES, pH 7.4, with NaOH, at the rate of 7–10 ml/min. Membrane current responses were low-pass filtered at 200 Hz, recorded onto DATs via a Bio-Logic DAT recorder (DTR1200), and simultaneously displayed on a Lectromed (Letchworth, UK) multitrace two-pen recorder. All drugs were applied via the superfusion system. The effects of indomethacin and Provera were assessed by using a concentration of GABA producing a half-maximal response (i.e., $EC_{50}$). Indomethacin and Provera were prepared as concentrated stock (1000×) in DMSO and diluted to the desired concentration in frog Ringer. The final maximum DMSO concentration (0.1%) had no effect on the control GABA-evoked

**Table 1. Summary of mIPSC parameters at 35°C from 62 DG and 57 CA1 neurons**

| | DG ($n = 62$) | CA1 ($n = 57$) |
|---|---|---|
| Peak amplitude (pA) | 71.57 ± 1.82 | 70.35 ± 1.8 |
| Rise time (msec) | 0.46 ± 0.01 | 0.47 ± 0.01 |
| T50 (msec) | 6.24 ± 0.67 | 5.63 ± 0.18 |
| T90 (msec) | 13.48 ± 0.34* | 12.11 ± 0.34* |
| $\tau$ (msec) | 7.54 ± 0.21 | 7.45 ± 0.23 |
| $\tau_1$ (msec) | 1.38 ± 0.17 | 1.1 ± 0.05 |
| $\tau_2$ (msec) | 11.49 ± 0.38 | 11.08 ± 0.37 |
| Cumulative $\tau$ (msec) | 7.46 ± 0.21 | 7.32 ± 0.22 |

*$p < 0.05$; CA1 versus DG; unpaired $t$ test.

response. Drugs were preapplied for 40–60 sec before being coapplied with the appropriate GABA concentration. Data were expressed as a percentage of the control response and statistically analyzed by repeated measures ANOVA.

## Results

As a prelude to investigate the role of neurosteroid metabolism in shaping GABAergic synaptic transmission, we first characterized the properties of $GABA_A$ receptor-mediated mIPSCs recorded at 35°C from hippocampal pyramidal CA1 and DG neurons. Such currents were reversibly blocked by the competitive $GABA_A$ receptor antagonist bicuculline (30 $\mu$M; data not shown). As illustrated in Table 1, CA1 and DG mIPSC parameters were similar and in agreement with previous studies on rats of similar age (Hollrigel and Soltesz, 1997; Harney et al., 2003).

### The effect of 5α3α and ganaxolone on mIPSCs recorded from CA1 and DG neurons

Previous investigations have suggested that $GABA_A$ receptors mediating synaptic transmission in hippocampal DG but not CA1 neurons are insensitive to the action of low (≤100 nM) concentrations of certain neurosteroids [e.g., 5α-tetrahydrodeoxycorticosterone (5α-THDOC) or 5β-pregnan-3α-ol-20-one], although higher steroid concentrations did enhance inhibitory transmission (Cooper et al., 1999; Harney et al., 2003). We, and others, have shown that the subunit composition and phosphorylation of the $GABA_A$ receptor or associated proteins influence neurosteroid action (Puia et al., 1993; Fancsick et al., 2000; Belelli et al., 2002; Harney et al., 2003; Koksma et al., 2003). However, these are unlikely to fully account for the differential neurosteroid sensitivity of dentate granule cells compared with CA1 neurons (see Discussion). An alternative, yet not mutually exclusive, explanation might lie in local steroid metabolism, which could blunt the steroid action in the dentate gyrus but not in the CA1 region. To test this hypothesis, we investigated and compared the actions of the most potent neurosteroid 5α3α and its metabolically stable synthetic analog ganaxolone on $GABA_A$ receptor-mediated mIPSCs recorded from CA1 and DG neurons. As shown in Figure 1, *A* and *B*, low concentrations (≤100 nM) of 5α3α significantly enhanced inhibitory transmission in CA1 neurons (i.e., the steroid prolonged the synaptic current decay of $GABA_A$ receptor-mediated mIPSCs in all cells tested; $p < 0.05$; KS test). Thus, 30 and 100 nM 5α3α significantly increased $\tau$ by 11 ± 3% ($n = 5$; $p < 0.05$) and 25 ± 5% ($n = 7$; $p < 0.01$), respectively (Table 2), whereas no significant effect was observed on the peak amplitude (data not shown). The synthetic derivative ganaxolone was similarly effective in CA1 neurons (Fig. 1*C*,*D*), with 30 and 100 nM of the steroid producing a significant increase of $\tau$ ($p < 0.01$ for both steroid concentrations) (Table 2) and no significant effect on the peak amplitude (data not shown). In contrast, when 5α3α action was investigated in the dentate gyrus,

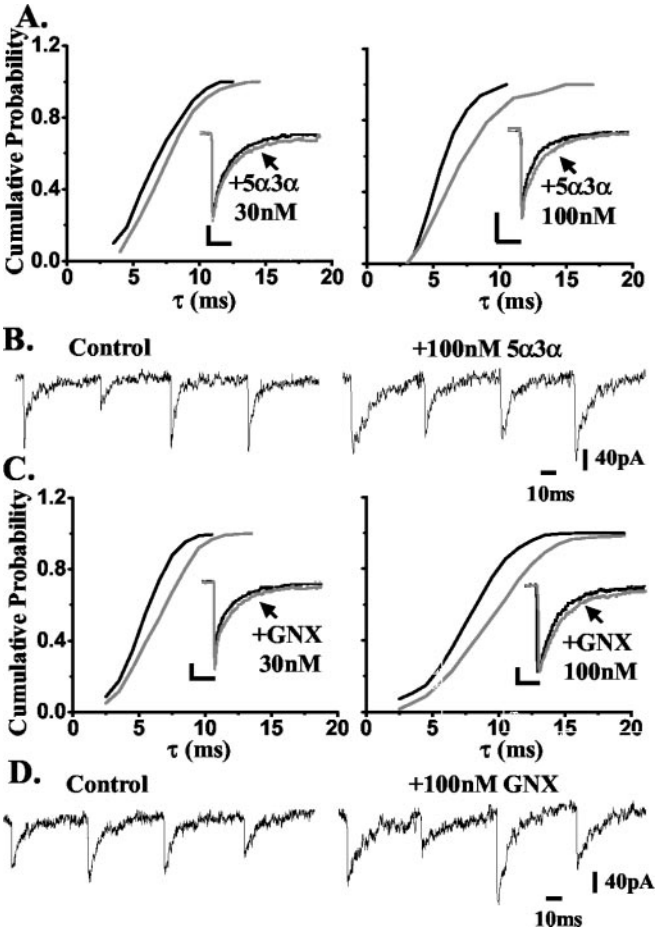


**Figure 1.** The effect of 5α3α and ganaxolone (GNX) on the decay of mIPSCs recorded from CA1 neurons. *A*, A cumulative probability plot of the decay of all mIPSCs, expressed as the cumulative time constant $\tau$ (see Materials and Methods), recorded from an exemplar CA1 neuron before and after the application of 30 (left) and 100 nM (right) 5α3α. The rightward shift of this relationship induced by either steroid concentration indicates that all mIPSCs recorded from this cell were sensitive to the neurosteroid. The insets illustrate the normalized ensemble average of all mIPSCs from the same CA1 neurons before and after application of 30 (left) and 100 nM (right) 5α3α. Calibration: 20 pA (left), 10 pA (right), 10 msec. *B*, Examples of individual mIPSC traces recorded from the same CA1 neuron before and after the application of 100 nM 5α3α. *C*, A cumulative probability plot of the decay of all mIPSCs, expressed as the cumulative time constant $\tau$ (see Materials and Methods), recorded from an exemplar CA1 neuron before and after the application of 30 (left) and 100 nM (right) ganaxolone. The rightward shift of this relationship induced by either steroid concentration indicates that all mIPSCs recorded from this cell were sensitive to this steroid. The insets illustrate the normalized ensemble average of all mIPSCs from the same CA1 neurons before and after application of 30 (left) and 100 nM (right) ganaxolone. Calibration: 10 pA, 10 msec. *D*, Examples of individual mIPSC traces recorded from the same CA1 neuron before and after the application of 100 nM ganaxolone.

**Table 2. The effect of 5α3α and ganaxolone on the decay time constant $\tau$ of mIPSCs recorded from CA1 and DG neurons**

| | CA1 | | Dentate gyrus | |
|---|---|---|---|---|
| $\tau$ Percentage increase | 5α3α | Ganaxolone | 5α3α | Ganaxolone |
| 30 nM | 11 ± 3%* $n = 5$ | 20 ± 3%**,*** $n = 5$ | 4 ± 3% $n = 7$ | 22 ± 4%**,*** $n = 7$ |
| 100 nM | 25 ± 5%** $n = 7$ | 25 ± 6%** $n = 6$ | 9 ± 4% $n = 7$ | 29 ± 9%**,*** $n = 6$ |

Relative changes are expressed as percentage increase of control values. *$p < 0.05$ versus control; **$p < 0.01$ versus control; ***$p < 0.05$ versus same concentration of 5α3α for a given neuronal type; repeated-measures ANOVA; $n$, number of neurons.

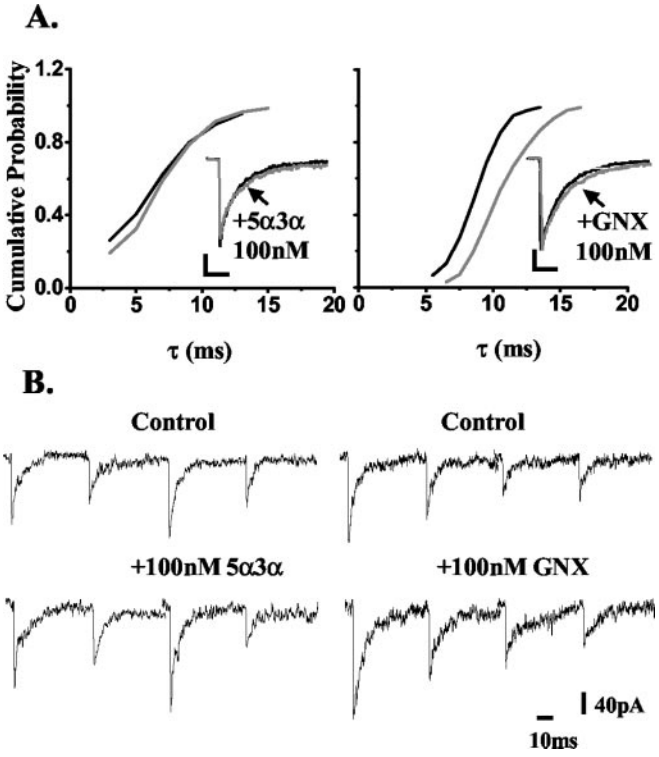


**Figure 2.** The effect of 5$\alpha$3$\alpha$ and ganaxolone (GNX) on the decay of mIPSCs recorded from DG neurons. *A*, A cumulative probability plot of the decay of all mIPSCs, expressed as the cumulative time constant $\tau$ (see Materials and Methods), recorded from an exemplar DG neuron before and after the application of 100 nM 5$\alpha$3$\alpha$ (left) and 100 nM ganaxolone (right). The rightward shift of this relationship induced by ganaxolone indicates that all mIPSCs recorded from this cell were sensitive to this steroid. In contrast, the lack of the rightward shift induced by 100 nM 5$\alpha$3$\alpha$ indicates that all mIPSCs recorded from this cell were insensitive to this neurosteroid. The insets illustrate the normalized ensemble average of all mIPSCs from the same DG neurons before and after application of 100 nM 5$\alpha$3$\alpha$ (left) and 100 nM ganaxolone (right). Calibration: 20 pA, 10 msec. *B*, Examples of individual mIPSC traces recorded from the same DG neurons before and after the application of 100 nM 5$\alpha$3$\alpha$ (left) and 100 nM ganaxolone (right).

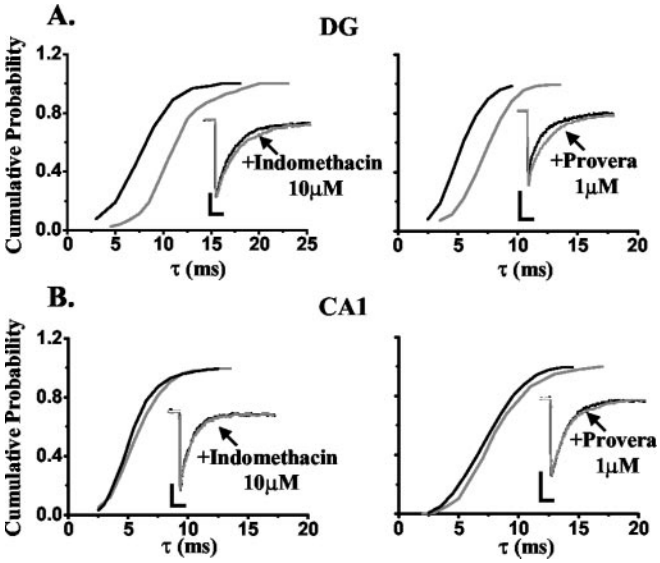


**Figure 3.** The effect of indomethacin and Provera on the decay of mIPSCs recorded from DG and CA1 neurons. *A*, A cumulative probability plot of the decay of all mIPSCs, expressed as the cumulative time constant $\tau$ (see Materials and Methods), recorded from an exemplar DG neuron before and after the application of 10 $\mu$M indomethacin (left) and 1 $\mu$M Provera (right). The rightward shift of this relationship induced by either indomethacin or Provera indicates that all mIPSCs recorded from these cells were sensitive to the 3$\alpha$-HSOR inhibitor. The insets illustrate the normalized ensemble average of all mIPSCs from the same DG neurons before and after application of 10 $\mu$M indomethacin (left) and 1 $\mu$M Provera (right). *B*, A cumulative probability plot of the decay of all mIPSCs, expressed as the cumulative time constant $\tau$ (see Materials and Methods), recorded from an exemplar CA1 neuron before and after the application of 10 $\mu$M indomethacin (left) and 1 $\mu$M Provera (right). The modest rightward shift of this relationship induced by either indomethacin or Provera indicates that all mIPSCs recorded from these cells were marginally sensitive to the 3$\alpha$-HSOR inhibitor. Note that the magnitude of the shift induced by either 3$\alpha$-HSOR inhibitor in the CA1 neuron is much smaller compared with that observed for a DG neuron. The insets illustrate the normalized ensemble average of all mIPSCs from the same CA1 neurons before and after application of 10 $\mu$M indomethacin (left) and 1 $\mu$M Provera (right). Calibration: 20 pA, 10 msec.

only 1 of 7 and 3 of 7 cells were sensitive to 30 and 100 nM of the steroid, respectively (KS test; $p < 0.05$). Overall, the changes in $\tau$ values were not significantly different from control for either concentration (Fig. 2*A*,*B*, left; Table 2) ( $p > 0.05$ for both concentrations). However, 30 and 100 nM ganaxolone prolonged the mIPSCs synaptic current decay of every DG neuron tested ($n = 6$–7; $p < 0.05$; KS test) (Fig. 2*A*,*B*, right; Table 2).

### The effect of inhibitors of 3$\alpha$-hydroxysteroidoxidoreductase on mIPSCs recorded from CA1 and DG neurons

It is clearly established that although the reduction of progesterone to 5$\alpha$-DHP by 5$\alpha$-reductase is irreversible, the reduction of the latter to 5$\alpha$3$\alpha$ by 3$\alpha$-HSOR can reverse to the inactive precursor (i.e., 5$\alpha$-DHP) (Mellon and Vaudry, 2001). Thus, we hypothesized that the differential sensitivity of DG granule cells to the action of 5$\alpha$3$\alpha$ versus ganaxolone may be accounted for by local steroid metabolism, that is, the endogenous progesterone metabolite would be oxidized back to the inactive precursor 5$\alpha$-dihydroprogesterone, whereas ganaxolone, by virtue of the methyl group in the 3$\beta$ position, would not (Carter et al., 1997). Thus, to test this hypothesis and investigate the contribution of local steroid metabolism to the selectivity of neurosteroid action, we used indomethacin (10 $\mu$M) to inhibit the activity of 3$\alpha$-HSOR. This concentration equates to five times the calculated $IC_{50}$ for indomethacin inhibition of rat brain 3$\alpha$-HSOR and, thus, is expected to completely block the activity of this enzyme (Penning et al., 1985). In a series of preliminary experiments, 10 $\mu$M indomethacin was devoid of action at recombinant $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes (response, 92 ± 3% of control; $n = 4$; data not shown). However, when the enzyme inhibitor was tested on mIPSCs recorded from DG neurons, the synaptic current decay (i.e., $\tau$) was significantly prolonged by 20 ± 4% ($n = 7$; $p < 0.05$) (Fig. 3*A*, left; Table 3). To validate the specificity of this effect, we also used the structurally distinct contraceptive agent Provera, which is reported to inhibit rat brain 3$\alpha$-HSOR with an ~10-fold greater potency than indomethacin (Penning et al., 1985). In common with indomethacin, Provera (1 $\mu$M) was devoid of action at recombinant $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes (response, 90 ± 5% of control; $n = 4$; data not shown) but significantly prolonged the synaptic current decay of mIPSCs recorded from DG neurons by 24 ± 5% ($n = 7$; $p < 0.05$) (Fig. 3*A*, right; Table 3). The increase in $\tau$ produced by 1 $\mu$M Provera was not significantly different from that caused by 10 $\mu$M indomethacin ( $p > 0.05$) (Table 3). Furthermore, in the presence of 1 $\mu$M Provera, 100 nM 5$\alpha$3$\alpha$ produced a significant increase of $\tau$ in addition to that produced by Provera or 5$\alpha$3$\alpha$ alone ( $p < 0.05$) (Table 3). Therefore, these results suggest that when metabolic inactivation is prevented, an endogenous neurosteroid tone is revealed, which is sufficient to modulate synaptic inhibition. Thus, these findings are consistent with the view that neurosteroid metabolism influ-

**Table 3. The effect of indomethacin and Provera on the decay time constant $\tau$ of mIPSCs recorded from CA1 and DG neurons**

| | Control | + Indomethacin (10 $\mu$M) | Control | + Provera (1 $\mu$M) | + Provera (1 $\mu$M) + 5$\alpha$3$\alpha$ (100 nM) | Control | + 5$\alpha$3$\alpha$ (100 nM) |
|---|---|---|---|---|---|---|---|
| Dentate gyrus | | | | | | | |
| $\tau$ (msec) | 7.8 ± 0.5 | 9.3 ± 0.7 | 7.1 ± 0.5 | 8.8 ± 0.6 | 9.7 ± 0.9 | 8.8 ± 1 | 9.5 ± 1 |
| | $n = 7$ | $n = 7$ | $n = 7$ | $n = 7$ | $n = 5$ | $n = 7$ | $n = 7$ |
| Percentage increase | | 20 ± 4*,** | | 24 ± 5*,** | 35 ± 6* | | 9 ± 4** |
| CA1 | | | | | | | |
| $\tau$ (msec) | 6.5 ± 0.5 | 6.8 ± 0.3 | 8.1 ± 0.5 | 9 ± 0.6 | | | |
| | $n = 5$ | $n = 5$ | $n = 8$ | $n = 8$ | | | |
| Percentage increase | | 7 ± 4*** | | 8 ± 2*,*** | | | |

Relative changes are expressed as percentage increase of control values. *$p < 0.001$ versus control; **$p < 0.05$ versus plus 1 $\mu$M Provera plus 100 nM 5$\alpha$3$\alpha$; ***$p < 0.05$ CA1 versus dentate gyrus; repeated-measures ANOVA; *n*, number of neurons.

ences $GABA_A$ receptor-mediated synaptic transmission in the dentate gyrus and, in addition, the selectivity of action of administrated neuroactive steroids.

The observation that, in contrast to the situation of the dentate gyrus, 5$\alpha$3$\alpha$ and ganaxolone are similarly effective at prolonging $GABA_A$ receptor-mediated mIPSCs in CA1 neurons suggests that 3$\alpha$-HSOR activity might display regional differences. To test this hypothesis, we evaluated and compared the actions of indomethacin and Provera on mIPSCs recorded from CA1 neurons. As illustrated in Table 3 and Figure 3*B*, 10 $\mu$M indomethacin had no significant effect (increase, 7 ± 4%; $n = 5$; $p > 0.05$) on CA1 mIPSCs, and 1 $\mu$M Provera produced only a modest but significant increase of $\tau$ (increase, 8 ± 2%; $n = 8$; $p < 0.05$). Furthermore, the effects of these 3$\alpha$-HSOR inhibitors in CA1 were significantly different from those observed in the DG ($p < 0.05$ for both indomethacin and Provera in CA1 vs dentate gyrus) (Table 3). These results imply that 3$\alpha$-HSOR activity differs between CA1 and dentate gyrus and, hence, influences the ability of administrated neuroactive steroids to modulate $GABA_A$ receptor-mediated neurotransmission in a manner that is neuron selective (see Discussion).

### The effect of 5$\alpha$3$\alpha$, ganaxolone, and Provera on the extrasynaptic $GABA_A$ receptor-mediated tonic conductance

A number of reports have provided compelling evidence for the presence in the dentate gyrus of an extrasynaptic $GABA_A$ receptor-mediated tonic conductance, which has been suggested to be mediated by receptors composed of $\alpha_4$,$\beta_x$ and $\delta$ subunits (Mody, 2001; Nusser and Mody, 2002; Stell and Mody, 2002). Recently, we and others have shown that this receptor combination is exquisitively sensitive to the actions of neurosteroids and to those of a number of therapeutically used drugs, which include the anesthetic agents etomidate and pentobarbital but not benzodiazepines (Belelli et al., 2002; Brown et al., 2002; Wohlfarth et al., 2002). Thus, we reasoned that if 3$\alpha$-HSOR enzymatic activity in the DG prevents modulation of synaptic $GABA_A$ receptors by exogenously applied 5$\alpha$3$\alpha$, this may additionally hinder modulation of the extrasynaptic $GABA_A$ receptor-mediated tonic conductance by 5$\alpha$3$\alpha$ but not by ganaxolone. Thus, in agreement with previous findings (Stell and Mody, 2002), application of bicuculline (30 $\mu$M) unmasked a tonic current of 32 ± 4 pA ($n = 17$). More importantly, 100 nM ganaxolone enhanced the tonic current in four of five cells tested from 21 ± 4 to 47 ± 7 pA (increase, 136 ± 28%; $p < 0.05$) (Fig. 4). In contrast, the tonic current from only one of five cells was sensitive to 5$\alpha$3$\alpha$ action with an increase of 147%. Overall, the tonic current in the presence of 100 nM 5$\alpha$3$\alpha$ was not significantly different from that calculated in its absence (control, 32 ± 8 pA; plus 100 nM 5$\alpha$3$\alpha$, 39 ± 8 pA; $p > 0.05$; $n = 5$) (Fig. 5). However, block of 3$\alpha$-HSOR by 1 $\mu$M Provera resulted in a significant increase of the tonic current from 40 ± 9 to 72 ± 18 pA (increase, 74 ± 8; $p < 0.05$; $n = 5$) (Fig. 6). The action of this contraceptive was blocked by the subsequent application of 30 $\mu$M bicuculline, thus demonstrating that the effects of Provera are $GABA_A$ receptor-mediated. Collectively, these results demonstrate that metabolic inactiva-

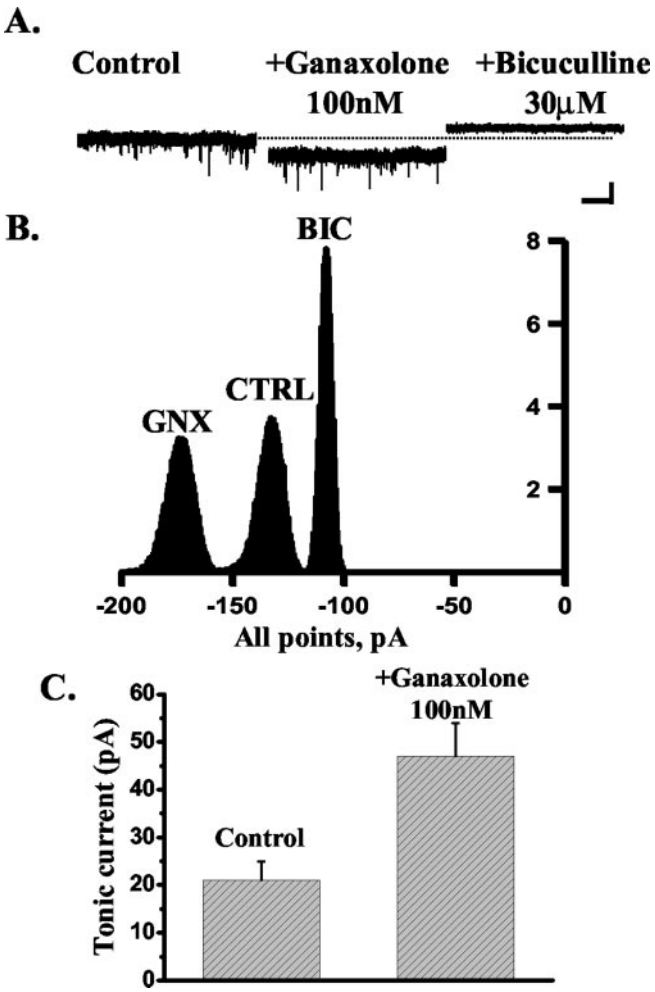


**Figure 4.** The effect of ganaxolone on the tonic current recorded from DG neurons. *A*, The tonic current, calculated as the difference between the holding current in the presence and absence of 30 $\mu$M bicuculline (see Materials and Methods), recorded from an exemplar DG neuron is enhanced by the application of 100 nM ganaxolone, and the steroid effect is blocked by the subsequent application of 30 $\mu$M bicuculline. The dashed line indicates the holding current (pA) under control conditions. Calibration: 50 pA, 5 sec. *B*, Corresponding all-point histograms illustrate the amplitude of the holding current under control conditions (CTRL) in the presence of 100 nM ganaxolone (GNX) and after the application of 30 $\mu$M bicuculline (BIC). *C*, Bar graph summarizing the enhancement of the tonic current induced by 100 nM ganaxolone in five DG neurons.

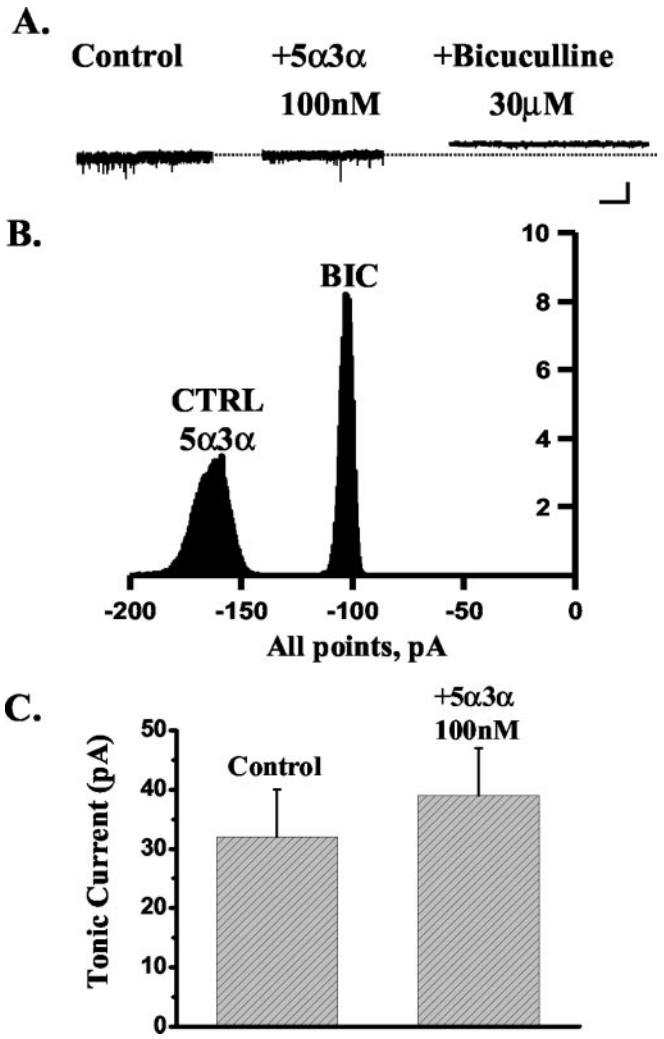


**Figure 5.** The effect of 5$\alpha$3$\alpha$ on the tonic current recorded from DG neurons. *A*, The tonic current, calculated as the difference between the holding current (pA) in the presence and absence of 30 $\mu$M bicuculline (see Materials and Methods), recorded from an exemplar DG neuron is insensitive to the application of 100 nM 5$\alpha$3$\alpha$. The dashed line indicates the holding current under control conditions. Calibration: 50 pA, 5 sec. *B*, Corresponding all-point histograms illustrate the amplitude of the holding current under control conditions (CTRL) in the presence of 100 nM 5$\alpha$3$\alpha$ and after the application of 30 $\mu$M bicuculline (BIC). Note that all-point histograms illustrating the amplitude of the holding current under control conditions and in the presence of 100 nM 5$\alpha$3$\alpha$ overlap, thus indicating the lack of sensitivity of the tonic current recorded from this cell to 5$\alpha$3$\alpha$ action. *C*, Bar graph summarizing the lack of a significant effect by 100 nM 5$\alpha$3$\alpha$ on the tonic current recorded from five DG neurons.

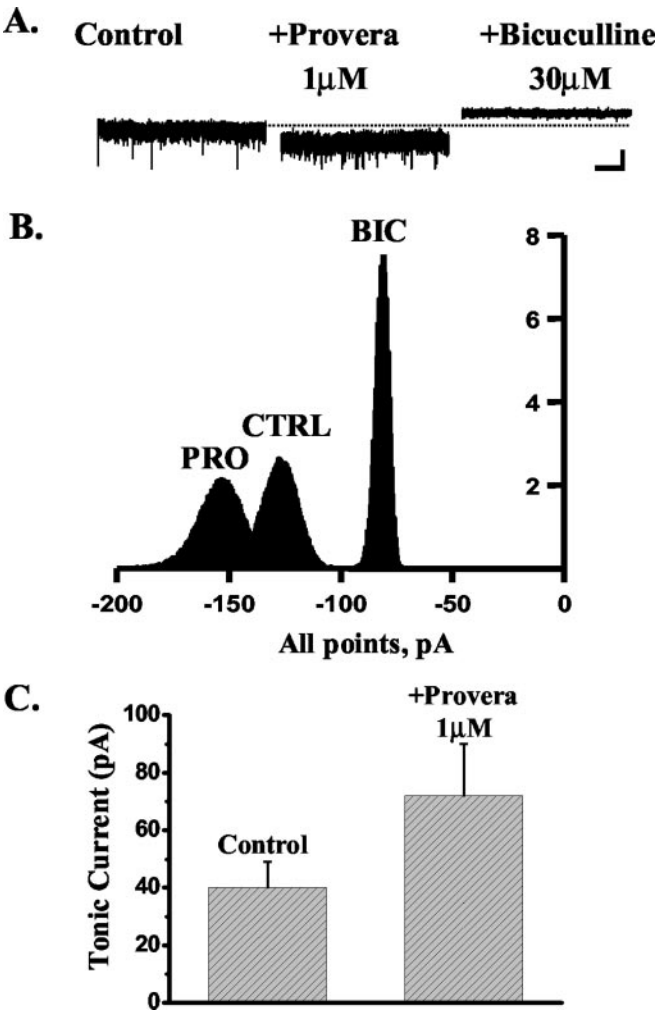


**Figure 6.** The effect of Provera on the tonic current recorded from DG neurons. *A*, The tonic current, calculated as the difference between the holding current (pA) in the presence and absence of 30 $\mu$M bicuculline (see Materials and Methods), recorded from an exemplar DG neuron is enhanced by the application of 1 $\mu$M Provera, and this effect is blocked by the subsequent application of 30 $\mu$M bicuculline. The dashed line indicates the holding current under control conditions. Calibration: 50 pA, 5 sec. *B*, Corresponding all-point histograms illustrate the amplitude of the holding current under control conditions (CTRL) in the presence of 1 $\mu$M Provera (PRO) and after the application of 30 $\mu$M bicuculline (BIC) *C*, Bar graph summarizing the enhancement of the tonic current induced by 1 $\mu$M Provera in five DG neurons.

tion of the endogenous neurosteroid tone in the dentate gyrus modulates inhibitory neurotransmission at both synaptic and extrasynaptic loci.

## Discussion

### The action of 5$\alpha$3$\alpha$ and ganaxolone on $GABA_A$ receptor-mediated inhibition in CA1 and dentate gyrus

The aim of the present investigation was twofold: (1) to evaluate the contribution of neurosteroid metabolism to the differential steroid sensitivity of neuronal $GABA_A$ receptors, and (2) to assess whether physiologically neurosteroid metabolism exerts a permissive role to shape $GABA_A$ receptor-mediated neurotransmission in a regionally dependent manner. To address the first objective, we compared and contrasted the action of the potent endogenously occurring progesterone metabolite 5$\alpha$3$\alpha$ and the metabolically stable 5$\alpha$3$\alpha$ analog, ganaxolone, on $GABA_A$ receptor-mediated mIPSCs recorded from CA1 and dentate granule cells. The findings presented here show that low concentrations of both 5$\alpha$3$\alpha$ and ganaxolone are similarly effective at prolonging mIPSCs recorded from CA1 neurons. These results are consistent with the observation that 5$\alpha$3$\alpha$ and ganaxolone are both equally effective and potent in enhancing the function of both recombinant and native $GABA_A$ receptors (Belelli et al., 1996a, b; Carter et al., 1997). In contrast, in the dentate gyrus, both synaptic and extrasynaptic $GABA_A$ receptors are sensitive to low concentrations of the synthetic analog ganaxolone but not to 5$\alpha$3$\alpha$. The relative insensitivity of DG synapses to 5$\alpha$3$\alpha$ is in agreement with previous reports on low concentrations ($\leq$100 nM) of either the 5$\beta$-isomer or 5$\alpha$-THDOC in brain slices prepared from rats of comparable age (Cooper et al., 1999; Harney et al., 2003). Proposed reasons for this relative insensitivity include receptor subunit composition and phosphorylation. Of the subunits expressed in dentate gyrus and CA1 that may impact on neurosteroid action (Nusser et al., 1996; Brooks-Kayal et al.,

2001), the $\alpha_1$ subunit and the $\alpha_4$ and δ-containing receptor assembly emerge as putative candidates to impart increased neurosteroid sensitivity. In contrast, receptors incorporating the $\alpha_2$ subunit are significantly less sensitive to physiologically relevant concentrations (≤100 nM) of 5α3α (Belelli et al., 2002; Brown et al., 2002). However, recombinant $GABA_A$ receptors containing any of these subunits do not discriminate between 5α3α and ganaxolone (Carter et al., 1997; Mascia et al., 2002; D. Belelli, unpublished observations). In addition, $\alpha_4$ and δ-containing recombinant receptors are exquisitively sensitive to steroid action.

Here, however, we demonstrate that extrasynaptic $GABA_A$ receptors in the dentate gyrus are relatively insensitive to 5α3α and, yet, such receptors are thought to comprise the $\alpha_4$ and δ subunits (Mody, 2001). Thus, it seems highly unlikely that the receptor subunit composition might explain the differential sensitivity of DG cells to 5α3α and ganaxolone. Recently, phosphorylation has been shown to impact on neurosteroid enhancement of synaptic inhibition (Fancsick et al., 2000; Vicini et al., 2002; Harney et al., 2003; Koksma et al., 2003). Although the precise mechanism by which such regulation occurs remains to be determined, it appears improbable that phosphorylation might selectively influence the actions of 5α3α but not those of ganaxolone. Collectively, these observations strongly implicate local metabolism in the differential sensitivity of DG cells to the endogenous and synthetic steroid. In rat brain, 5α3α can be metabolized by the following different pathways: (1) it can be reduced at the 20 position to the potent $GABA_A$ receptor-active neurosteroid 5α-pregnan-3α,20α-diol (Belelli et al., 1996b), (2) it can be sulfated at the 3α-OH group, and (3) it can be oxidized back to the 5α3α precursor (i.e., 5α-DHP). The latter two metabolites are devoid of action at $GABA_A$ receptors with the conversion of 5α3α to 5α-DHP being the most important pathway at least in the brain (Korneyev et al., 1993; Dong et al., 2001). In contrast, ganaxolone, by virtue of the methyl group in the 3β position, potentially can only be converted to the 20 hydroxy $GABA_A$ receptor-acting metabolite or possibly the 3α-sulfate ester. Contrary to 5α3α, the anticonvulsant action of ganaxolone in both animals and humans is relatively long lasting (Carter et al., 1997; Monaghan et al., 1997). Thus, although a detailed pharmacokinetic profile of ganaxolone in the rodent brain is not available, either the steroid is in the main metabolically stable or the primary metabolites must be active at the $GABA_A$ receptor.

**The role of 3α-HSOR**

The findings discussed above suggest that local neurosteroid metabolism can impart selectivity to the action of administrated steroids at the $GABA_A$ receptor. Furthermore, they imply that local metabolism of endogenous neurosteroids influences $GABA_A$ receptor-mediated inhibition in a neuron-specific manner. To validate this hypothesis, we blocked the activity of 3α-HSOR in both CA1 and DG neurons with indomethacin and confirmed the specificity of this effect with a more potent, structurally diverse inhibitor of 3α-HSOR (i.e., Provera). Although both ligands display additional pharmacological actions (e.g., indomethacin inhibits arachidonate cyclo-oxygenase, whereas Provera is active at cytosolic progesterone receptors) (Goodman et al., 2001), such actions are unlikely to account for the rapid prolongation of the mIPSC decay or enhancement of $GABA_A$ receptor-mediated extrasynaptic tonic conductance observed in dentate gyrus. In addition, neither agent exhibits a direct positive modulatory action on recombinant $GABA_A$ receptors.

The reaction catalyzing the conversion of 5α-DHP to 5α3α is established as reversible (Mellon and Vaudry, 2001). Specifically, Li et al. (1997) have identified in rat brain two isoforms of 3α-HSOR: one subtype that is cytosolic and primarily catalyzes the reaction in the reductive direction (i.e., leading to the formation of 5α3α), and another isoform that is membrane-bound and catalyzes the reaction preferentially in the oxidative direction (i.e., leading to the formation of 5α-DHP). Interestingly, when these two enzymatic activities were examined across the rodent brain, a differential regional distribution became apparent, and the dentate gyrus in particular was shown to have a high activity of the membrane-bound 3α-HSOR isoform (Li et al., 1997). We speculate that in the dentate gyrus, the membrane-bound isoform of 3α-HSOR regulates the endogenous levels of 5α3α. This proposal is consistent with previous reports showing that conversion of nanomolar concentrations of 5α3α and 5α-THDOC to 5α-DHP and 5α-deoxycorticosterone, respectively, occurs in rat brain (Purdy et al., 1991). Furthermore, $^3$H-5α3α is rapidly metabolized to 5α-DHP in a neuroblastoma cell line, and this conversion can be blocked by indomethacin (Rupprecht et al., 1993). Whether the locus of enzyme action is intracellular remains to be determined. From the evidence presented here, it is argued that 3α-HSOR activity in dentate gyrus is sufficient to prevent modulation of neuronal synaptic and extrasynaptic inhibition by relatively low concentrations of exogenously applied 5α3α. Furthermore, the results with either indomethacin or Provera suggest that if not for metabolic inactivation, endogenous dentate gyrus levels of 5α3α would be high enough to enhance both synaptic and extrasynaptic $GABA_A$ receptor-mediated inhibition. Interestingly, the magnitude of the steroid effect on the decay of mIPSCs recorded from the dentate gyrus when 3α-HSOR activity is blocked is similar to that produced by the same steroid concentration at CA1 synapses in the absence of inhibition of 3α-HSOR. These findings, coupled with the observation that inhibition of 3α-HSOR activity in CA1 has little if any effect on synaptic inhibition, suggest that the naturally occurring levels of 5α3α are low in both CA1 and dentate gyrus but for different reasons. In the dentate gyrus, local 5α3α is metabolically inactivated by 3α-HSOR, whereas in CA1, the steroid levels are maintained low by other means, possibly because of a reduced synthesis.

To our knowledge, these results provide the first demonstration that 3α-HSOR may shape neuronal inhibition mediated by both synaptic and extrasynaptic $GABA_A$ receptors in a neuron-specific manner. In addition, this enzyme influences the action of exogenously applied 5α3α. Such findings are consistent with the recent report that IPSCs recorded from cortical neurons of *in vitro* brain slices obtained from mice injected systemically with a 5α-reductase inhibitor (i.e., SKF 105111) display faster decay kinetics than their vehicle-injected controls (Puia et al., 2003). Collectively, these observations imply that physiological and pathophysiological alterations of neurosteroid anabolic or catabolic machinery might selectively alter neuronal inhibition. In support of this view, in a mouse model of psychiatric disorders, abnormally low levels of 5α3α are positively correlated with a reduced 5α-reductase activity (Dong et al., 2001). It is tempting to speculate that some human neurological and psychiatric illnesses associated with inhibitory dysfunction and abnormal levels of $GABA_A$ receptor-acting neurosteroids (e.g., catamenial epilepsy and postpartum dysphoria) might also be characterized by regionally dependent alterations of the steroid metabolic machinery.

In summary, we demonstrated that selectivity of neurosteroid action at inhibitory GABAergic synaptic and extrasynaptic sites is influenced by local steroid metabolism in a neuron-specific man-

ner. In this regard, we speculate that the ability of the contraceptive agent Provera to inhibit 3$\alpha$-HSOR might have important neurological implications on the long-term use of this progestin and the action of androgen and progesterone derivatives on inhibitory centers in the CNS. It is intriguing that administration of medroxyprogesterone (i.e., Provera) has long been known to be therapeutically beneficial for the treatment of catamenial seizures but not of noncatamenial epilepsy (Newmark and Penry, 1980). These observations highlight 3$\alpha$-HSOR as a potential novel target for the development of new therapeutics in the treatment of catamenial epilepsy. Importantly, these discoveries provide the starting point to investigate the role played by specific enzymes involved in neurosteroid metabolism in pathophysiological conditions associated with inhibitory dysfunction.

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