0026-895X/95/030603-08$3.00/0

MOLECULAR PHARMACOLOGY, 47:603–610, 1995.

Chronic Neurosteroid Treatment Produces Functional Heterologous Uncoupling at the γ-Aminobutyric Acid Type A/ Benzodiazepine Receptor Complex in Mammalian Cortical Neurons

RONG YU and MAHARAJ K. TICKU

Department of Pharmacology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284–7764

Received July 14, 1994; Accepted January 2, 1995

SUMMARY

We have investigated the effects of chronic treatment with the neurosteroid 5α-pregnan-3α-ol-20-one (5α3α) on the γ-aminobutyric acid $(GABA)_A$ receptor complex in cultured mammalian cortical neurons. Chronic 5α3α treatment (up to 2 μM, 5 days) did not produce any changes in the morphological appearance or the cell protein content of cortical neurons. The basal binding of [^{3}H]flunitrazepam, [^{3}H]Ro15–1788, and [^{3}H]Ro15–4513 was not altered after the chronic treatment. Chronic 5α3α treatment did not alter the K_d or B_{max} values of [^{3}H]flunitrazepam binding to intact cortical neurons. However, chronic 5α3α treatment produced uncoupling between GABA, barbiturate, and neurosteroid sites and the benzodiazepine site. The EC_{50} values of these ligands were not significantly altered; however, their E_{max} values were decreased after chronic 5α3α treatment. The 5α3α-induced uncoupling was time and concentration dependent. The binding of [^{3}H]GABA and *t*-[^{35}S]butylbicyclophosphorothionate was also decreased after chronic 5α3α treatment. Chronic 5α3α treatment decreased the B_{max} of the low affinity $GABA_A$ receptor sites, without affecting the high affinity sites, and decreased the B_{max} of *t*-butylbicyclophosphorothionate binding sites. The EC_{50} value for GABA-induced $^{36}Cl^-$ influx was not altered, whereas the E_{max} value was decreased after chronic 5α3α treatment. Furthermore, the 5α3α-induced uncoupling was reversed by concomitant exposure of the cortical neurons to 5α-pregnan-3β-ol-20-one or R5135, suggesting an involvement of the neurosteroid and GABA recognition sites in the observed uncoupling. Taken together, these results suggest that chronic 5α3α treatment produces heterologous uncoupling at the $GABA_A$ receptor complex.

The $GABA_A$ receptor is a member of a gene superfamily of ligand-gated ion channels that includes nicotinic acetylcholine, glutamate, and glycine receptors (1). The $GABA_A$ receptor protein is a hetero-oligomeric protein complex with a GABA recognition site, a chloride ion channel, and at least four other sites for the action of modulatory drugs such as picrotoxin, barbiturates, neurosteroids, and benzodiazepines (2).

The discovery of a biosynthetic pathway for steroids in oligodendrocytes (3) provided evidence for the synthesis of neurosteroids from cholesterol. Since then, many studies have been performed to try to elucidate the significance of these neurosteroids, a term referring to steroids of central origin that are independent of peripheral sources. It was more than half a century ago that Seyle (4) observed that steroids exhibited anesthetic and anticonvulsant effects. There is evidence that steroids can influence neuronal excitability and that some endogenous neurosteroids may play a role in the regulation of central nervous system excitabilty (5). It was observed that exposure of male rats to brief swim stress at ambient temperature elevated the levels of 5α3α (allopregnanolone) and 3α,21-dihydroxy-5α-pregnan-20-one (allotetrahydrodeoxycorticosterone or tetrahydrodeoxycorticosterone), which are metabolites of progesterone and deoxycorticosterone, respectively, in cerebral cortex and hypothalamus within 5 min (6). The elevated levels of these two neurosteroids were high enough to modulate GABA receptors, because these neurosteroids interact with [^{35}S]TBPS and [^{3}H]GABA binding sites associated with the $GABA_A$ receptor complex. Those authors also found that allopregnanolone could be measured in brain tissue from adrenalectomized male rats and female adrenalectomized and oopho-

This work was supported by National Institutes of Health Grant NS15339.

ABBREVIATIONS: GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TBPS, *t*-butylbicyclophosphorothionate; Ro15–1788, ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5α][1,4]benzodiazepine-3-carboxylate; Ro15–4513, ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5α][1,4]benzodiazepine-3-carboxylate; 5α3α, 5α-pregnan-3α-ol-20-one; 5α3β, 5α-pregnan-3β-ol-20-one; 5β3α, 5β-pregnan-3α-ol-20-one; R5135, 3α-hydroxy-16-imino-5β,17-androstan-11-one; MEM, minimal essential medium.

rectomized rats. These data support the notion that these steroids are synthesized *de novo* in brain and may function as endogenous modulators of certain receptors in the central nervous system.

Several lines of evidence suggest that neurosteroids modulate the activity of the $GABA_A$ receptor-chloride ionophore complex (7–13). The 3α-hydroxy, ring A-reduced, steroids 5α3α and tetrahydrodeoxycorticosterone were found to be active ligands of the $GABA_A$ receptor complex, with affinities equal to or greater than those of the benzodiazepines (14). Furthermore, these neuroactive steroids have been proposed to be endogenous modulators of central $GABA_A$ receptors (7).

The evidence for the existence of a high affinity neurosteroid modulatory site on the $GABA_A$ receptor is now compelling (15, 16). The potent and stereoselective modulation of the $GABA_A$ receptor by neurosteroids (9, 17), together with the observation that intracellularly applied neurosteroids are inactive (18), suggests that there is a specific steroid binding site associated with the $GABA_A$ receptor complex.

In contrast to the *in vitro* studies, the effects of chronic neurosteroid treatment on the $GABA_A$ receptor have received limited attention. 5β3α is a reduced derivative of progesterone that potently enhances both the GABA-induced Cl^- current and the binding of [^{3}H]flunitrazepam to the $GABA_A$ receptor (8, 9). A recent study indicated that chronic treatment (10 μM, 48 hr) with 5β3α and 5α3α eliminated the potentiation by 5β3α of [^{3}H]flunitrazepam binding in chick whole-brain neurons (19). In this study, we have further investigated the effect of chronic 5α3α treatment on the $GABA_A$ receptor complex in mammalian cultured cortical neurons using radioligand binding and $^{36}Cl^-$ influx studies. We have conducted our studies under precisely controlled conditions and independently of pharmacokinetic variability, in well characterized mammalian cortical neurons (20).

Experimental Procedures

Coverslip preparation. Before plating, 25-mm round coverslips were bent on the edge for handling with forceps. Ten sterile plastic coverslips were placed in a 100-mm polystyrene dish, to which a sterile solution of 0.1 M boric acid (pH 8.4 with NaOH) and poly-L-lysine (1 mg/100 ml) was added. The coverslips were soaked in this solution overnight and then rinsed with nutrient medium, pH 7.4 (MEM 10/10), which contained 80% Eagle's MEM, 33.3 mM glucose, 26.2 mM $NaHCO_3$, 10% heat-inactivated (56° for 30 min) horse serum, and 10% fetal bovine serum. Each coverslip was placed in a 35-mm tissue culture Petri dish containing 1 ml of MEM 10/10; then they were placed in an incubator with 95% air/5% CO_2 at 37°.

Cell culture. Embryos from 14-day-old C57BL/6CR mice were removed from their sacs and transferred to a 35-mm culture Petri dish containing ice-cold aerated (95% O_2/5% CO_2) Puck's buffer, pH 7.4 (100 ml of 10× Puck's saline, 10 ml of 1 M HEPES, and 50 ml of 12% glucose/30% sucrose solution; <320–330 mOsmol). Using a microscope with a light source, the cerebral hemispheres were removed from a single embryo with iridectomy scissors and placed in another 35-mm Petri dish containing ice-cold aerated Puck's buffer. The tissue was then minced with iridectomy scissors in an empty 60-mm Petri dish and soaked in nutrient medium (MEM 10/10), pH 7.4. The tissue fragments were transferred to a sterile tube for trituration. This cycle of resuspension in MEM 10/10 and trituration was repeated until the supernatant volume was 2 ml/embryo. These dissociated cells were plated on poly-L-lysine-coated, sterile, 25-mm, round coverslips by addition of 0.5 ml of suspension to dishes containing 1 ml of MEM 10/10 that had been preincubated with 95% air/5% CO_2 at 37° for at least 1 hr.

After the plated cells had been incubated for 24 hr, 1 ml of nutrient medium was replaced with 1 ml of medium containing 10% heat-inactivated horse serum only (MEM 10), and a mixture of sterile 5-fluoro-2′-deoxyuridine and uridine (2 mg/ml 5-fluoro-2′-deoxyuridine and 5 mg/ml uridine), at a final concentration of 10 μg/ml, was added. A portion (1 ml) of the medium was replaced with MEM 10 after 3 days. For chronic pentobarbital and neurosteroid treatment studies, freshly prepared solutions of pentobarbital sodium and neurosteroid were added to the culture medium. For some experiments the cells were grown in culture flasks, using an identical procedure.

Binding studies. Coverslips containing cultured cortical neurons were removed from the tissue culture medium and rinsed three times (5 min each) at room temperature in HEPES-buffered saline (136 mM NaCl, 5.4 mM KCl, 1.2 mM $MgCl_2$, 1 mM NaH_2PO_4, 20 mM HEPES, adjusted to pH 7.4 with Tris base). Triplicate coverslips containing neurons were then incubated with [^{3}H]flunitrazepam (1 nM), [^{3}H]Ro15–1788 (1 nM), or [^{3}H]Ro15–4513 (1 nM), with or without other drugs, in HEPES-buffered saline, pH 7.4, for 30 min at 24°. For saturation experiments, the concentration of [^{3}H]flunitrazepam was varied between 0.25 and 20 nM. Nonspecific binding was determined in parallel in the presence of 10^{-5} M Ro15–1788. After incubation, the coverslips were rapidly transferred to a beaker containing 1000 ml of ice-cold HEPES-buffered saline solution, followed by immersion for 7 sec in HEPES-buffered saline in another beaker. Each coverslip was drained on tissue paper and transferred to a scintillation vial containing 1.5 ml of 0.2 N NaOH. After 1 hr, a 0.5-ml aliquot was removed for protein determination and the remaining solution was neutralized with 1 N HCl (200 ml), mixed with 10 ml of Hydrofluor, and counted by liquid scintillation counting.

For [^{35}S]TBPS and [^{3}H]GABA binding studies, a mitochondrial/microsomal (P_2 plus P_3) fraction was prepared, as described (20–22). Briefly, cultured neurons were scraped from the flasks, homogenized in cold 0.32 M sucrose with a Teflon/glass homogenizer, and centrifuged at 100 × *g* for 10 min. The supernatant was centrifuged at 140,000 × *g* for 30 min to obtain the mitochondrial/microsomal (P_2 plus P_3) fraction. This fraction was resuspended in ice-cold double-distilled water and homogenized with a Brinkman Polytron homogenizer, at a setting of 6, in 14-sec bursts. The suspension was centrifuged at 140,000 × *g* for 30 min, and the pellet was washed three times by homogenization and centrifugation with buffer ([^{35}S]TBPS binding, 200 mM KCl, 5 mM Tris·HCl, pH 7.4; [^{3}H]GABA binding, 50 mM KCl, 50 mM Tris·HCl, pH 7.4) and was then frozen. On the day of the assay, the tissue was thawed, similarly centrifuged, washed two more times, and resuspended in Tris buffer. For initial experiments, aliquots of membrane suspension were incubated with 10 nM [^{35}S]TBPS for 180 min or with 4 nM [^{3}H]GABA for 10 min, at room temperature. [^{35}S]TBPS binding was measured by a filtration assay (21) and [^{3}H]GABA binding was measured by a centrifugation assay, as described (22). Briefly, for GABA binding studies, aliquots of control and treated membrane suspensions were incubated with [^{3}H]GABA (4 nM), in the presence or absence of excess nonradioactive GABA (10^{-4} M), in a total incubation volume of 1 ml for 10 min. After incubation, the vials were centrifuged at 48,000 × *g* for 10 min. The vials were washed and solubilized, and the radioactivity was determined as described (22). For Scatchard analysis, the concentration of [^{3}H]GABA was varied up to 4 nM; concentrations between 4 nM and 1004 nM were subjected to radioisotopic dilution. Routinely, 12 concentrations of [^{3}H]GABA were utilized for saturation experiments. For [^{35}S]TBPS saturation experiments, the concentration was varied between 1 and 205 nM. Nonspecific binding was determined in the presence of 10^{-4} M picrotoxinin for [^{35}S]TBPS and 10^{-4} M GABA for [^{3}H]GABA binding.

$^{36}Cl^-$ influx studies. Control and chronically neurosteroid-treated coverslips with attached cultures were removed from the tissue culture medium, rinsed three times (5 min each) at room temperature in HEPES-buffered saline solution, pH 7.4, and drained rapidly on tissue paper, followed by immediate transfer to 2 ml of

HEPES-buffered saline containing $^{36}Cl^-$ (2 μCi/ml), in the absence or presence of various drugs. GABA-mediated $^{36}Cl^-$ influx was measured in the presence of the uptake blocker nipecotic acid (10^{-4} M). Influx was terminated after 5 sec by rapid transfer of the coverslip with attached cultures to another beaker containing 1000 ml of ice-cold stop solution (150 mM NaCl, 5.4 mM KCl, 1.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1 mM NaH_2PO_4, 5 mM HEPES, adjusted to pH 7.4 with Tris base), with immersion for 7 sec, as described (23, 24). After this 7-sec immersion in the freshly made stop solution, each coverslip was drained on tissue paper, transferred to a scintillation vial containing 1.5 ml of 0.2 N NaOH, and processed as described for the binding studies.

Drugs that were insoluble in water were dissolved in dimethylsulfoxide and used at a final dimethylsulfoxide concentration of ≤0.1%. Control experiments demonstrated that dimethylsulfoxide at up to 0.1% did not have any effect on radioligand binding.

Data analysis. Protein was estimated by the bicinchoninic acid protein assay (25). All values for radioligand binding are expressed as femtomoles/milligram of protein. EC_{50} and E_{max} values were derived with Delta Graph Professional version 2.0.2 software (Macintosh), using curve fitting. The K_d and B_{max} values were obtained with the same program, using either linear regression (for TBPS and flunitrazepam) or curve fitting (user-defined) (for GABA). All data were analyzed, where appropriate, by one-way analysis of variance.

Results

Effects of chronic neurosteroid 5α3α treatment on cortical neurons. The protein content for coverslips treated with 5α3α (up to 2 μM) for 5 days was similar to that of control. Results from five independent representative experiments are presented in Table 1. This indicates that 5α3α treatment did not affect the cellular protein content of cortical neurons. Furthermore, chronic 5α3α treatment (2 μM, 5 days) did not affect the morphological characteristics of cortical neurons, as visualized by phase-contrast microscopy (data not shown). The concentration of 5α3α (2 μM) used for these studies was established in preliminary experiments. To establish that the cells were not damaged by the neurosteroid treatment, we measured the equilibrium $^{36}Cl^-$ uptake in control and 5α3α (2 μM, 5 days)-treated cells. Preliminary studies indicated that $^{36}Cl^-$ attained equilibrium in 15 min. The equilibrium $^{36}Cl^-$ uptake (30-min incubation) was 11.33 ± 2.2 nmol/mg of protein (three experiments, each done in triplicate) in control cells and 11.05 ± 1.9 nmol/mg of protein (three experiments, each done in triplicate) in 5α3α-treated cells. These results indicate that chronic 5α3α treatment did not make cells leaky.

TABLE 1

Effect of chronic neurosteroid 5α3α treatment (2 μM, 5 days) on total cellular protein content in cultured cortical neurons

Coverslips containing neurons were rinsed, as described in Experimental Procedures, before measurement of protein content. Results are from five independent experiments. The values are the mean ± standard deviation of triplicate determinations.

Experiment	Protein	
	Control group	Treated group
	μg/coverslip	
1	128 ± 13	133 ± 10
2	150 ± 9	143 ± 37
3	159 ± 10	163 ± 14
4	125 ± 9	127 ± 21
5	133 ± 29	131 ± 20

The cultured cortical neurons used in the present study contain all of the components of the $GABA_A$/benzodiazepine receptor ionophore complex (20). In our initial studies, we examined the effect of chronic 5α3α treatment on the basal binding of various benzodiazepine ligands to the benzodiazepine receptor site. Table 2 shows that chronic 5α3α treatment (2 μM, 5 days) did not alter the basal binding of [^{3}H]flunitrazepam (agonist), [^{3}H]Ro15–1788 (antagonist), or [^{3}H]Ro15–4513 (inverse agonist) to the intact cortical neurons. Further analysis of saturation isotherms revealed that chronic 5α3α treatment (2 μM, 5 days) did not alter the K_d or B_{max} values of [^{3}H]flunitrazepam binding (Table 3). These results also establish that there is no residual steroid in our cultures, because 5α3α enhanced [^{3}H]benzodiazepine agonist binding *in vitro* (data not shown).

Effects of chronic 5α3α treatment on coupling/uncoupling of the $GABA_A$ receptor complex. After 5α3α treatment (2 μM, 5 days), the specific [^{3}H]flunitrazepam binding was not altered; however, there was a significant decrease in the enhancement of [^{3}H]flunitrazepam binding by GABA, pentobarbital sodium, and neurosteroid 5α3α. Fig. 1 shows the concentration-dependent enhancement of [^{3}H]flunitrazepam binding by GABA (Fig. 1A), pentobarbital sodium (Fig. 1B), and 5α3α (Fig. 1C) in control and chronically treated cortical neurons. Studies of the enhancement by various modulators of [^{3}H]flunitrazepam binding showed that, whereas the EC_{50} values of GABA, pentobarbital sodium, and 5α3α were unaltered, their E_{max} values were decreased after chronic 5α3α treatment (Table 4). The chronic neurosteroid treatment produced maximal uncoupling between GABA and benzodiazepine binding sites (75%). The rank order of uncoupling was GABA > pentobarbital = 5α3α. All subsequent experiments were performed to further characterize the uncoupling between GABA and benzodiazepine sites. The effect of chronic 5α3α treatment on the uncoupling of GABA and benzodiazepine sites was time (Fig. 2) and concentration (Fig. 3) dependent.

Effects of chronic 5α3α treatment on [^{3}H]GABA and [^{35}S]TBPS binding. Our initial preliminary studies, using single-point analysis, indicated that chronic 5α3α treatment decreased the binding of both GABA and TBPS to their recognition sites. To further characterize this effect, we analyzed the saturation isotherm data by Scatchard analysis. [^{3}H]GABA bound to two sites in the cortical neuronal membranes, which is consistent with published reports. Table 5 shows the effect of chronic 5α3α treatment on the K_d and B_{max} values for GABA binding to cortical neurons. Chronic 5α3α treatment decreased the B_{max} of the low affinity

TABLE 2

Effect of chronic neurosteroid 5α3α treatment (2 μM, 5 days) on the specific binding of [^{3}H]flunitrazepam, [^{3}H]Ro15–1788, and [^{3}H]Ro15–4513 in intact cortical neurons

The values are the mean ± standard deviation of three separate experiments, each performed in triplicate.

Radioligand (1 nM)	Specific binding	
	Control group	Treated group
	fmol/mg of protein	
[^{3}H]Flunitrazepam	140 ± 9	140 ± 11
[^{3}H]Ro15–1788	144 ± 1	141 ± 7
[^{3}H]Ro15–4513	143 ± 4	145 ± 6

TABLE 3

Effect of chronic 5α3α treatment (2 μM, 5 days) on the K_d and B_{max} values for [^{3}H]flunitrazepam binding to intact cortical neurons

[^{3}H]Flunitrazepam (0.5–20 nM) binding to intact cortical neurons was measured as described in Experimental Procedures. The Scatchard plots were analyzed by linear regression to obtain K_d and B_{max} values. The values represent the mean ± standard deviation of three experiments.

Group	[^{3}H]Flunitrazepam binding	
	K_d	B_{max}
	nM	*fmol/mg of protein*
Control	3.4 ± 0.7	824 ± 113
Chronic 5α3α	4.1 ± 0.8	726 ± 47

$GABA_A$ receptor sites, without altering the K_d or the B_{max} for the high affinity site.

[^{35}S]TBPS bound to a single site in cortical neurons, with an apparent K_d value of 47 ± 11 nM and a B_{max} value of 1840 ± 155 fmol/mg of protein (four experiments). Chronic 5α3α treatment (2 μM, 5 days) decreased the B_{max} value of [^{35}S]TBPS binding to 1368 ± 117 fmol/mg of protein (Table 6).

Effects of chronic 5α3α treatment on GABA-induced $^{36}Cl^-$ influx. To investigate the effect of chronic 5α3α treatment on the functional aspects of GABA-ergic transmission, we measured the GABA-mediated $^{36}Cl^-$ influx in these neurons. Chronic 5α3α treatment did not alter the basal $^{36}Cl^-$ levels (control, 2.506 ± 0.12 nmol/mg of protein; chronic 5α3α treatment, 2.413 ± 0.03 nmol/mg of protein; mean ± standard deviation, three experiments). GABA produced a concentration-dependent increase in $^{36}Cl^-$ influx, with an EC_{50} value of 10.8 ± 1.1 μM and maximal enhancement of 84 ± 2% in control neurons (Fig. 4; Table 7). In chronically treated neurons, the E_{max} value of GABA was decreased to 26 ± 2%, whereas the EC_{50} value was not altered (Fig. 4; Table 7). The results indicated that chronic neurosteroid treatment produced a decreased efficacy of GABA receptor-mediated functional response in the cortical neurons.

Reversal by 5α3β and R5135 of chronic 5α3α-induced uncoupling. To investigate the possible mechanisms of 5α3α-induced uncoupling, we examined the effects of concomitant exposure of cortical neurons to 5α3α and its inactive isomer, 5α3β (2 μM), or the competitive $GABA_A$ antagonist receptor R5135 (1 μM). In contrast to the active isomer 5α3α (e.g., Fig. 1C), the inactive isomer 5α3β did not enhance basal [^{3}H]flunitrazepam binding (data not shown). The chronic 5α3α-induced uncoupling was reversed by concomitant exposure of neurons to either 5α3β (Fig. 5) or R5135 (Fig. 6).

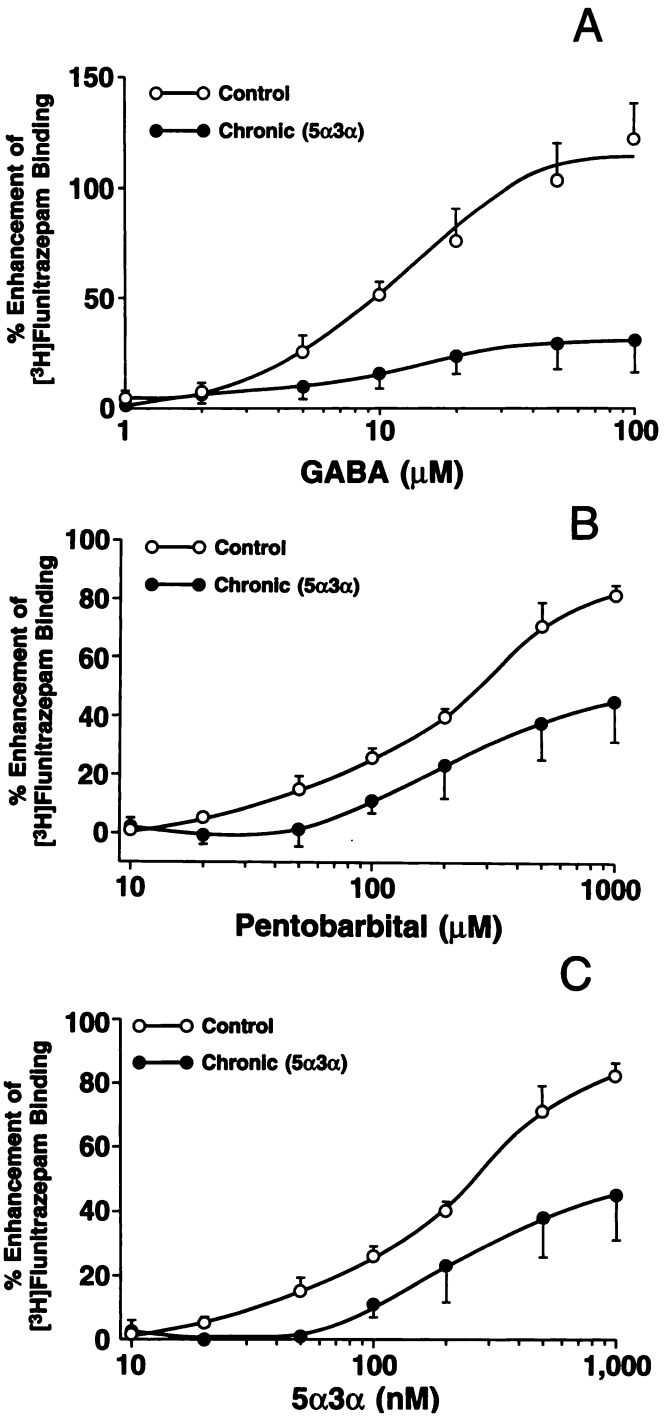

Fig. 1. Concentration-dependent enhancement of [^{3}H]flunitrazepam (1 nM) binding by GABA (A), pentobarbital sodium (B), and 5α3α (C) in control and neurosteroid (2 μM 5α3α, 5 days)-treated cortical neurons. The values are the mean ± standard deviation of three separate experiments, each done in triplicate.

Discussion

Steroid hormones are synthesized from cholesterol. Gluco- and mineralocorticosteroids are synthesized in the adrenal glands, and sex steroids are synthesized in the gonads and the placenta (e.g., see Ref. 26). In the central nervous system, steroid hormones influence the function of many nerve cells. For example, in the hypothalamus there are neurons that secrete hypophysiotropic factors that stimulate or inhibit the production of pituitary hormones such as adrenocorticotropic hormone and gonadotropins. These neurons are under the regulation of their corresponding steroid hormones and are under a feedback control mechanism (26). Although we are beginning to understand how steroids influence these neurons in the hypothalamus, not much is known regarding how these steroid hormones influence mental, behavioral, and metabolic processes.

In recent years, it has become apparent that many of the steroid-induced changes occur rapidly (e.g., see Ref. 5), suggesting possible nongenomic effects. There is evidence showing that 5α3α (allopregnanolone), a metabolite of progester-

TABLE 4

Effect of chronic neurosteroid 5α3α treatment (2 μM, 5 days) on the EC_{50} and E_{max} values for GABA, pentobarbital, and 5α3α enhancement of [^{3}H]flunitrazepam binding in cortical neurons

Values are the mean ± standard deviation of three to five separate experiments, each done in triplicate. Values in parentheses represent percentage of uncoupling.

Modulator	[^{3}H]Flunitrazepam binding			
	EC_{50}		E_{max}	
	Control group	Treated group	Control group	Treated group
	μM		%	
GABA	14 ± 3	12 ± 6	130 ± 28	37 ± 9 (−72%)[a]
Pentobarbital	213 ± 29	241 ± 15	93 ± 8	48 ± 11 (−48%)[a]
5α3α	0.150 ± 0.05	0.131 ± 0.01	73 ± 6	44 ± 8 (−40%)[a]

[a] $p < 0.001$, compared with the control group.

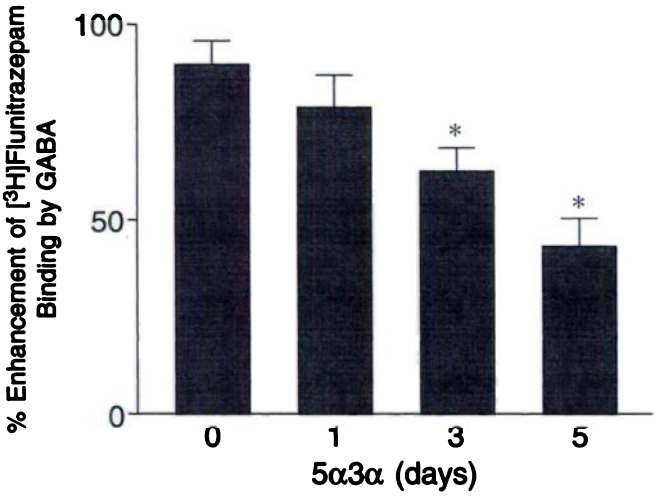

Fig. 2. Enhancement by GABA (500 μM) of [^{3}H]flunitrazepam (1 nM) binding to intact control neurons and neurons treated with the neurosteroid 5α3α (2 μM) for 1, 3, or 5 days. The values are the mean ± standard deviation of three separate experiments, each done in triplicate.

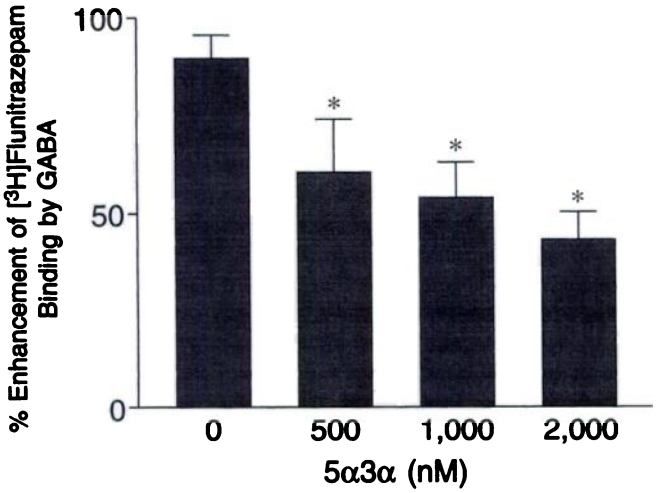

Fig. 3. Enhancement by GABA (500 μM) of [^{3}H]flunitrazepam (1 nM) binding to intact control neurons and neurons treated with various concentrations of the neurosteroid 5α3α for 5 days. The values are the mean ± standard deviation of three separate experiments, each done in triplicate.

one, is synthesized *de novo* in the brain (6). Early structure-activity studies showed that there was no correlation between the polarity of the steroids and their potency, and

TABLE 5

Effect of chronic 5α3α treatment (2 μM, 5 days) on [^{3}H]GABA binding to cortical neurons

Membranes were treated as described in Experimental Procedures. GABA Scatchard plots were determined using 12 concentrations of [^{3}H]GABA (0.25–1004 nM). The K_d and B_{max} values were determined by curve fitting (user defined) (Delta Graph; Macintosh). The values represent the mean ± standard deviation of three or four experiments.

Group	Specific [^{3}H]GABA binding			
	K_d		B_{max}	
	K_{d_1}	K_{d_2}	B_{max_1}	B_{max_2}
	nM		fmol/mg of protein	
Control	3.4 ± 1.5	131 ± 29	347 ± 110	2457 ± 222
Chronic 5α3α	4.3 ± 2.6	107 ± 35	343 ± 28	1602 ± 319[a]

[a] $p < 0.02$, compared with the control group.

TABLE 6

Effect of chronic 5α3α treatment (2 μM, 5 days) on [^{35}S]TBPS binding to cortical neurons

Membranes were prepared as described in Experimental Procedures. [^{35}S]TBPS Scatchard plots were analyzed by linear regression to obtain K_d and B_{max} values. The values represent the mean ± standard deviation of three or four experiments.

Group	[^{35}S]TBPS binding	
	K_d	B_{max}
	nM	fmol/mg of protein
Control (four experiments)	47 ± 11	1840 ± 155
Chronic 5α3α (three experiments)	45 ± 10	1368 ± 117[a]

[a] $p < 0.01$, compared with the control group.

the 3β-hydroxy isomer was shown to be inactive (17). Several lines of evidence suggest that neurosteroids modulate $GABA_A$-ergic transmission (7–14). Recent evidence obtained with recombinantly expressed $GABA_A$ receptors argues strongly in favor of a distinct steroid modulatory site on the $GABA_A$ receptor (15).

Our results demonstrated that chronic neurosteroid treatment, although it did not alter the basal binding of benzodiazepine ligands, produced a heterologous uncoupling between GABA, barbiturate, and neurosteroid sites and the benzodiazepine receptor site. Our results showing that chronic neurosteroid treatment did not alter benzodiazepine binding (K_d or B_{max}) are consistent with a previous report (19). Additionally, the same treatment produced a down-regulation of GABA and TBPS binding sites and a functional uncoupling between the GABA recognition site and the chloride channels. Chronic neurosteroid treatment produced a decrease in the B_{max} of the lower affinity $GABA_A$ receptor

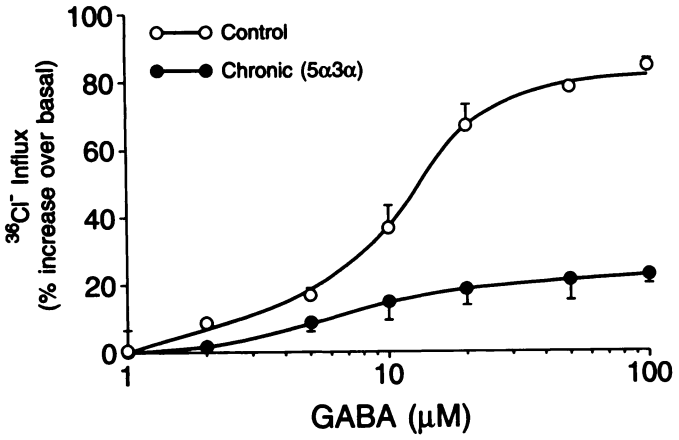

Fig. 4. Concentration-dependent enhancement of GABA-induced $^{36}Cl^-$ influx in control and chronically 5α3α (2 μM, 5 days)-treated cortical neurons. The values are the mean ± standard deviation of three separate experiments, each done in triplicate.

TABLE 7

Effect of chronic neurosteroid treatment (2 μM, 5 days) on GABA-induced $^{36}Cl^-$ influx in cortical neurons

GABA (1–100 μM)-induced $^{36}Cl^-$ influx was measured in control and chronically treated neurons, as described in the text. Values represent the mean ± standard deviation of three separate experiments, each done in triplicate.

	$^{36}Cl^-$ influx			
	EC_{50}		E_{max}	
	Control group	Treated group	Control group	Treated group
	μM		%	
GABA	10.8 ± 1.1	11.7 ± 4.9	84 ± 2	26 ± 2 (−69%)[a]

[a] $p < 0.001$, compared with the control group.

sites. Our previous studies have shown that low, but not high, affinity GABA receptor sites are coupled to the benzodiazepine sites (e.g., see Ref. 22). Like GABA receptor binding, chronic 5α3β treatment decreased the B_{max} of [^{35}S]TBPS binding, without altering the K_d value. Thus, there was a parallel down-regulation of GABA and TBPS binding. The observation that chronic neurosteroid treatment down-regulated GABA and TBPS binding without altering benzodiazepine binding is intriguing and needs further investigation. However, based on what is known in the literature regarding $GABA_A$ receptor subunits and their expression, it could be speculated that αβ subunits are needed for GABA and TBPS binding, whereas αγ2 subunits are needed for benzodiazepine binding. Thus, if chronic neurosteroid treatment decreased the relative levels of β subunits, the net result could be down-regulation of GABA/TBPS binding, without an alteration in the benzodiazepine binding, an effect observed in our study. However, this is only speculative at this time and needs to be investigated.

The ability of the stereoisomer 5α3β and R5135, a competitive $GABA_A$ receptor antagonist, to reverse the uncoupling suggests an involvement of the neurosteroid and GABA recognition sites in these events. The ability of the stereoisomer 5α3β to reverse the uncoupling may be due to its ability to occupy the neurosteroid site. Our results are, in part, consistent with a report in which chronic 5β3α and 5α3α treatment (10 μM, 48 hr) was reported to produce uncoupling between the steroid and benzodiazepine binding sites in chick whole-

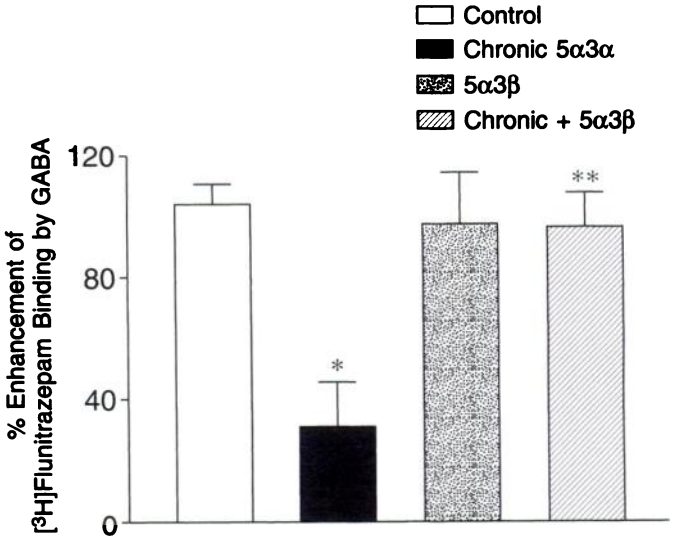

Fig. 5. Effect of chronic 5α3α treatment (2 μM, 5 days), and its reversal by concomitant exposure of cortical neurons to 5α3β (2 μM, 5 days), on GABA enhancement of [^{3}H]flunitrazepam (1 nM) binding. The values are the mean ± standard deviation of three separate experiments. *, $p < 0.05$, compared with the control group; **, $p < 0.05$, compared with the 5α3α- plus 5α3β-treated group.

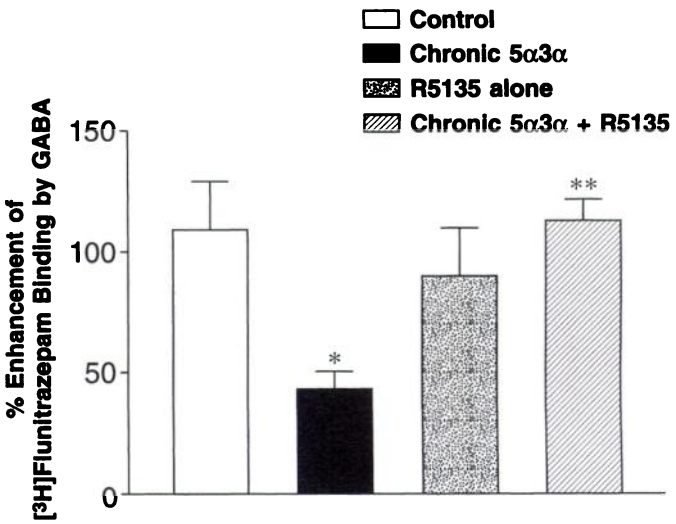

Fig. 6. Effect of chronic 5α3α treatment (2 μM, 5 days), and its reversal by concomitant exposure of cortical neurons to R5135 (1 μM, 5 days), on GABA enhancement of [^{3}H]flunitrazepam (1 nM) binding. The values are the mean ± standard deviation of three separate experiments. *, $p < 0.05$, compared with the control group; **, $p < 0.05$, compared with the chronically 5α3α- plus R5135-treated group.

brain neurons (19). However, those investigators observed complete uncoupling (100%), and this effect was not reversed by the $GABA_A$ receptor antagonist SR-95531. In our study, maximal uncoupling of 75% was observed between GABA and benzodiazepine sites, and only 45% uncoupling was observed between the neurosteroid or barbiturate sites and the benzodiazepine site. Furthermore, in our study, the uncoupling between GABA and benzodiazepine sites was reversed by both the $GABA_A$ receptor antagonist R5135 and the stereoisomer 5α3β. These differences could be due to differences in the concentrations of neurosteroid (2 versus 10 μM), the time of chronic treatment (5 days versus 48 hr), or the tissue used to prepare cultured neurons (mammalian cortical neu-

rons versus chick whole-brain neurons). The ability of R5135 to reverse uncoupling suggests that a tonic GABA effect may be required for neurosteroid-induced uncoupling.

In previous studies, it was reported that chronic GABA treatment produced down-regulation of the GABA receptor complex and uncoupling (20, 27, 28). Functional studies using either $^{36}Cl^-$ influx or electrophysiological recording showed that chronic GABA treatment produced a decreased efficacy of GABA-induced responses (20, 28). Our recent studies also demonstrated that chronic GABA treatment produced a decrease in the steady state mRNA levels and a decrease in the polypeptide levels of the α2 (53-kDa) and α3 (59-kDa) subunits of the $GABA_A$ receptor in cortical neurons (29). Down-regulation of GABA receptor subunit mRNAs after treatment with $GABA_A$ receptor agonists has also been reported by others (30, 31). In other studies, we previously observed that chronic benzodiazepine agonist treatment did not alter the binding associated with the $GABA_A$ receptors but it produced uncoupling and decreased the efficacy of benzodiazepine ligand modulation of GABA-induced $^{36}Cl^-$ influx (32, 33). Thus, it appears that various ligands that modulate $GABA_A$-ergic transmission may differentially alter GABA receptor binding, coupling, and function after chronic treatment.

The potential molecular mechanisms involved in uncoupling and decreased efficacy are not known. From the cloning and expression studies, it is clear that the α variants are crucial in determining the degree of coupling between GABA and benzodiazepine sites and benzodiazepine potentiation of GABA responses in transfected cells (34–36). These studies have demonstrated that the α3 subunit gives maximal enhancement by GABA of benzodiazepine agonist binding and maximal efficacy of benzodiazepine agonists in enhancing GABA-ergic responses (34–36). We speculate that chronic treatment of the $GABA_A$ receptor complex with various modulators could produce an alteration in the levels of various α subunits that form the functional $GABA_A$ receptor isoforms. Such an alteration in the levels of various α subunits could produce functionally different $GABA_A$ receptor isoforms and may be an underlying mechanism for uncoupling and/or decreased efficacy. However, the possible involvement of other mechanisms, such as post-translational modification, in these events cannot be ruled out.

Finally, our studies demonstrating that chronic neurosteroid treatment may alter the efficacy of GABA-ergic transmission have physiological consequences. The concentration of the neurosteroid used in our study is similar to that reported to alter $GABA_A$ receptor binding and function (8–13), and similar concentrations have been observed during the estrus cycle and pregnancy in rats (37). Plasma and brain levels of 5α3α were found to closely follow the levels of the precursor progesterone (37, 38). During pregnancy, the maternal plasma concentration of 5α3α was found to be high, because its precursor progesterone was synthesized at an increased rate in the placenta and fetal tissue (39). Because 5α3α has been demonstrated to have hypnotic and anxiolytic effects (40, 41), a change in its concentration, as well as $GABA_A$ receptor subsensitivity after exposure to high concentrations of 5α3α during pregnancy, may contribute to changes in mood and increased somnolence during pregnancy and anxiety and depression during the postpartum period. Similar steroid-induced $GABA_A$ receptor subsensitivity may occur during the menstrual cycle. High levels of reduced metabolites of progesterone during the luteal phase (42) may result in the development of autodependency on this natural anxiolytic and may contribute to symptoms of premenstrual anxiety.

Acknowledgments

The authors thank Mrs. Sadie Phillips for excellent secretarial assistance.

References

1. Schofield, P. R., M. G. Darlison, N. Fujita, D. R. Burt, F. A. Stephenson, H. Rodriquez, L. M. Rhee, J. Ramachandran, V. Reale, T. A. Glencorse, P. H. Seeburg, and E. A. Barnard. Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor superfamily. *Nature (Lond.)* **328**:221–227 (1988).
2. Olsen, R. W., and J. C. Venter. *Receptor Biochemistry and Methodology, Vol. 5, Benzodiazepine/GABA Receptors and Chloride Channels: Structural and Functional Properties*. Alan R. Liss, New York (1986).
3. Baulieu, E. E. Steroid hormones in the brain: several mechanisms? in *Steroid Hormone Regulation of the Brain* (K. Fuxe, J. A. Gustafsson, and L. Wetterberg, eds.). Pergamon Press, New York, 3–14 (1981).
4. Seyle, H. The antagonism between anesthetic steroid hormone and pentamethylenetetrazole (metrazol). *J. Lab. Clin. Med.* **27**:1051–1053 (1942).
5. McEwen, B. J. Non-genomic effects of steroids in neural activity. *Trends Pharmacol. Sci.* **12**:141–147 (1991).
6. Purdy, R. H., A. L. Morrow, P. H. Moore, and S. M. Paul. Stress-induced elevation of γ-aminobutyric acid type A receptor-active steroids in the rat brain. *Proc. Natl. Acad. Sci. USA* **88**:4553–4557 (1991).
7. Majewska, M. D., N. L. Harrison, R. D. Schwartz, J. L. Barker, and S. M. Paul. Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science (Washington D. C.)* **232**:1004–1007 (1986).
8. Harrison, N. L., and M. A. Simmonds. Modulation of the GABA receptor complex by a steroid anesthetic. *Brain Res.* **323**:287–292 (1984).
9. Harrison, N. L., M. D. Majewska, J. W. Harrington, and J. L. Barker. Structure-activity relationships for steroid interaction with the $GABA_A$ receptor complex. *J. Pharmacol. Exp. Ther.* **241**:346–353 (1987).
10. Gee, K. W., W. C. Chang, R. E. Brinton, and B. S. McEwen. GABA-dependent modulation of the Cl^- ionophore by steroids in rat brain. *Eur. J. Pharmacol.* **136**:419–423 (1987).
11. Gee, K. W., M. B. Bolger, E. Brinton, H. Coirini, and B. S. McEwen. Steroid modulation of chloride ionophore in rat brain: structure-activity requirements, regional dependence and mechanism of action. *J. Pharmacol. Exp. Ther.* **246**:803–812 (1988).
12. Morrow, L. A., J. R. Pace, R. H. Purdy, and S. M. Paul. Characterization of steroid interaction with γ-aminobutyric acid receptor-gated chloride ion channels: evidence for multiple steroid recognition sites. *Mol. Pharmacol.* **37**:263–270 (1990).
13. Turner, D. M., R. W. Ransom, J. S.-J. Yang, and R. W. Olsen. Steroid anesthetics and naturally occuring analogs modulate the γ-aminobutyric acid receptor complex at a site distinct from barbiturates. *J. Pharmacol. Exp. Ther.* **248**:960–966 (1989).
14. Paul, S. M., and R. H. Purdy. Neuroactive steroids. *FASEB J.* **6**:2311–2322 (1992).
15. Puia, G., M. R. Santi, S. Vicini, D. Pritchett, R. H. Purdy, S. M. Paul, P. H. Seeburg, and E. Costa. Neurosteroids act on recombinant human $GABA_A$ receptors. *Neuron* **4**:759–765 (1990).
16. Lan, N. C., K. W. Gee, M. B. Bolger, and J. S. Chen. Differential responses of expressed recombinant human $GABA_A$ receptors to neurosteroids. *J. Neurochem.* **57**:1818–1821 (1991).
17. Cottrell, G. A., J. J. Lambert, and J. A. Peters. Modulation of $GABA_A$ receptor activity by alphaxalone. *Br. J. Pharmacol.* **90**:491–500 (1987).
18. Lambert, J. J., J. A. Peters, N. C. Sturgess, and T. G. Hales. Steroid modulation of the $GABA_A$ receptor complex: electrophysiological studies. *Ciba Found. Symp.* **153**:56–71 (1990).
19. Friedman, L., T. T. Gibbs, and D. H. Farb. γ-Aminobutyric $acid_A$ receptor regulation: chronic treatment with pregnanolone uncouples allosteric interactions between steroid and benzodiazepine recognition sites. *Mol. Pharmacol.* **44**:191–197 (1993).
20. Mehta, A. K., and M. K. Ticku. Chronic GABA exposure down-regulates GABA-benzodiazepine receptor-ionophore complex in cultured cerebral cortical neurons. *Mol. Brain Res.* **16**:29–36 (1992).
21. Maksay, G., and M. K. Ticku. The dissociation of [^{35}S]*t*-butylbicyclophosphorothionate binding differentiates convulsant and depressant drugs that modulate GABAergic transmission. *J. Neurochem.* **44**:480–486 (1985).
22. Burch, T. P., R. Thyagarajan, and M. K. Ticku. Group-selective reagent modification of the benzodiazepine-γ-aminobutyric-acid receptor-ionophore complex reveals that low-affinity γ-aminobutyric acid receptors stimulate benzodiazepine binding. *Mol. Pharmacol.* **23**:52–59 (1983).
23. Mehta, A. K., and M. K. Ticku. Ethanol potentiation of GABAergic trans-

mission in cultured spinal cord neurons involves $GABA_A$-gated chloride channels. *J. Pharmacol. Exp. Ther.* **246:**558–564 (1988).
24. Mehta, A. K., and M. K. Ticku. Benzodiazepine and *beta*-carboline interactions with $GABA_A$ receptor-gated chloride channels in mammalian cultured spinal cord neurons. *J. Pharmacol. Exp. Ther.* **249:**418–423 (1989).
25. Redinbaugh, M. G., and R. B. Turley. Adaptation of the bicinchoninic acid protein assay for use with microtiter plates and sucrose gradient fractions. *Anal. Biochem.* **153:**267–271 (1986).
26. Berne, R. M., and M. N. Levy. *Physiology*, Ed. 3. St. Louis, Mosby Year Book (1993).
27. Hablitz, J. J., M. H. Jalilian Tehrani, and E. M. Barnes, Jr. Chronic exposure of developing cortical neurons to GABA downregulates GABA/benzodiazepine receptors and GABA-gated chloride currents. *Brain Res.* **501:**332–338 (1989).
28. Roca, D. J., I. Rozenberg, M. Farrant, and D. H. Farb. Chronic agonist exposure induces down-regulation and allosteric uncoupling of the γ-aminobutyric acid/benzodiazepine receptor complex. *Mol. Pharmacol.* **37:**37–43 (1990).
29. Mhatre, M. C., and M. K. Ticku. Chronic GABA treatment downregulates the $GABA_A$ receptor α_2 and α_3-subunit mRNAs as well as polypeptide expression in primary cultured cerebral cortical neurons. *Mol. Brain Res.* **24:**159–165 (1994).
30. Montpeid, P., E. I. Ginns, B. M. Martin, D. Roca, D. H. Farb, and S. M. Paul. γ-Aminobutyric acid (GABA) induces a receptor-mediated reduction in $GABA_A$ receptor α subunit messenger RNAs in embryonic chick neurons in culture. *J. Biol. Chem.* **266:**6011–6014 (1991).
31. Hirouchi, M., D. Seitaro, and K. Kuriyama. Muscimol-induced reduciton of $GABA_A$ receptor α_1-subunit mRNA in primary cultured cerebral cortical neurons. *Mol. Brain Res.* **15:**327–331 (1991).
32. Hu, X.-J., and M. K. Ticku. Chronic benzodiazepine treatment produces functional uncoupling of the γ-aminobutyric acid-benzodiazepine receptor ionophore complex in cortical neurons. *Mol. Pharmacol.* **45:**618–625 (1994).
33. Hu, X.-J., and M. K. Ticku. Chronic flurazepam treatment produces decreased efficacy of the benzodiazepine ligands and pentobarbital with γ-aminobutyric $acid_A$ receptor in cortical neurons. *J. Pharmacol. Exp. Ther.* **270:**485–490 (1994).
34. Pritchett, D., H. Sonthheimer, B. D. Shivers, S. Ymer, H. Kettenmann, P. R. Schofield, and P. Seeburg. Importance of a novel $GABA_A$ receptor unit for benzodiazepine pharmacology. *Nature (Lond.)* **338:**582–585 (1989).
35. Pritchett, D. B., H. Luddens, and P. H. Seeberg. Type I and type II $GABA_A$-benzodiazepine receptor produced in transfected cells. *Science (Washington D. C.)* **245:**1389–1392 (1989).
36. Puia, G., S. Vicini, P. H. Seeburg, and E. Costa. Influnce of recombinant γ-aminobutyric $acid_A$ receptor subunit composition on the action of allosteric modulators of γ-aminobutyric acid-gated Cl^- currents. *Mol. Pharmacol.* **39:**691–696 (1991).
37. Ishikawa, S., T. Sawada, Y. Nakamura, and T. Marioka. Ovarian secretion of pregnane compounds during estrous cycle and pregnancy in rats. *Endocrinology* **94:**1615–1620 (1974).
38. Kraulis, I., G. Foldes, H. Traikov, B. Dubrovsky, and M. K. Birmingham. Distribution, metabolism and biological activity of deoxycorticosterone in the central nervous system. *Brain Res.* **88:**1–14 (1975).
39. Milewich, L., V. Kaimal, and A. R. Johnson. Steroid 5α-reductase activity in human placenta. *Am. J. Obstet. Gynecol.* **133:**611–617 (1987).
40. Crawley, J. N., J. R. Glowa, M. D. Majewska, and S. M. Paul. Anxiolytic activity of endogenous adrenal steroid. *Brain Res.* **339:**382–386 (1986).
41. Holzbauer, M. Physiological aspects of steroids with anesthetic properties. *Med. Biol.* **22:**97–102 (1976).
42. Rosciszewska, D., B. Buntner, I. Guz, and L. Zawisza. Ovarian hormones, anticonvulsant drugs, and seizures during the menstrual cycle in women with epilepsy. *J. Neurol. Neurosurg. Psychiatry* **49:**47–51 (1986).

Send reprint requests to: Maharaj K. Ticku, Department of Pharmacology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78284–7764.