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Anxiolytic Effect of Progesterone is Associated With Increases in Cortical Allopregnanolone and $GABA_A$ Receptor Function

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BITRAN, D., R. H. PURDY AND C. K. KELLOGG. *Anxiolytic effect of progesterone is associated with increases in cortical allopregnanolone and $GABA_A$ receptor function.* PHARMACOL BIOCHEM BEHAV **45**(2) 423–428, 1993.—The effects of a SC injection of progesterone (0, 1, or 4 mg) on locomotor behavior and exploration of an elevated plus-maze were examined in ovariectomized rats. At the completion of the behavioral tests, blood serum and cerebral cortical level of the 3α-hydroxy ring-A metabolite of progesterone, 3α-hydroxy-5α-pregnan-20-one (allopregnanolone), was also assessed. GABA-stimulated $^{36}Cl^-$ influx was studied in cortical synaptoneurosomes from a subgroup of ovariectomized females treated with vehicle or 4 mg progesterone. Whereas progesterone treatment did not affect ambulation in a novel arena, significant anxiolytic behavior was detected in the plus-maze 4 h after administration of 1 or 4 mg progesterone. A dose-dependent increase in allopregnanolone level was found in serum and cortical homogenates. Studies of GABA-stimulated Cl^- influx demonstrated that progesterone treatment increased the sensitivity of cortical synaptoneurosomes to GABA (i.e., decreased the EC_{50}) and increased the maximal efficacy with which GABA stimulated Cl^- transport (i.e., increased the E_{max}). Together, these data support the hypothesis that the psychotropic effects observed after progesterone administration are due to the bioconversion of progesterone to allopregnanolone, which subsequently augments $GABA_A$ receptor-mediated function.

Neurosteroid 3α-Hydroxy-5α-pregnan-20-one Anxiety Elevated plus-maze $^{36}Cl^-$ influx Synaptoneurosomes Ovariectomy

ADRENAL and gonadal steroids are known to affect the functions of the CNS via intracellular steroid receptors and subsequent modification of protein synthesis (18,19). Most of the steroid-induced genomic effects occur in neural structures governing reproductive function or the activity of the hypothalamic-pituitary axis. In addition to these genomic effects, some steroids alter the excitability of the CNS via rapid nongenomic interaction with membrane-bound neurotransmitter receptors (36). Hans Selye long ago described sedative/anesthetic effects of progesterone and its natural ring-A reduced metabolite, 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) (37,38). Only recently has the mechanism underlying the potent anesthetic property of neuroactive steroids been elucidated (21,22). Pharmacological, biochemical, and electrophysiological studies demonstrated that 3α-hydroxy ring-A reduced metabolites of progesterone and deoxycorticosterone directly interact with the GABA-gated chloride ion channel ($GABA_A$ receptor) [reviewed in (2,10,26)]. These steroids display a high affinity for the $GABA_A$ receptor complex and potentiate chloride ion (Cl^-) conductance in a manner similar to the barbiturates.

The in vivo effects of pregnane steroids parallel the well-documented in vitro effects reviewed above. IV administration of progesterone or iontophoretic application of allopregnanolone potentiated GABA-induced hyperpolarization of cerebellar Purkinje neurons in vivo (40). Both effects were blocked by coadministration of bicuculline (39), a $GABA_A$ receptor antagonist. Systemic administration of allopregnanolone was found to protect against bicuculline-induced seizures (1). Behavioral studies in animals have shown dose-dependent and stereospecific analgesic (14), anxiolytic (3,7,41), antiaggressive (13), and sedative/hypnotic effects (20,25) following administration of allopregnanolone or allotetrahydrodeoxycorticosterone, a neuroactive steroid metabolized from deoxycorticosterone.

The experiments described herein were designed to assess whether progesterone exerts anxiolytic and/or sedative effects and determine if progesterone's putative effects are mediated by its bioconversion to alloprenanolone. The rationale for these studies is found in previous clinical findings reporting an inverse relationship between seizure susceptibility and plasma levels of progesterone and allopregnanolone in catamenial epi-

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lepsy (24,34,35). In a recent study of healthy human female subjects, progesterone administration resulted in plasma levels of allopregnanolone that were correlated with increased fatigue and confusion and decreased verbal memory and motor coordination (E. W. Freeman and R. H. Purdy, personal communication). Notably, the progesterone-induced effects bear a striking similarity to effects observed following benzodiazepine administration (5,16). In animal studies, progesterone administration in ovariectomized estrogen-primed rats was found to increase punished responding in a conflict paradigm (i.e., feeding that was paired to electric shock), a measure of anxiolytic efficacy (33). More recently, systemic progesterone injection in ovariectomized rats was noted to elicit potent antiaggressive effects that were correlated to an inhibition of amygdaloid and hippocampal ^{35}S-*t*-butyl-bicyclophosphorothionate (^{35}S-TBPS) binding (6), a ligand thought to bind on or near the GABA-gated Cl^- ionophore.

We now report that systemic administration of progesterone in ovariectomized rats increased exploration of an elevated plus-maze. In addition, the behavioral effects were correlated with cerebral cortical levels of allopregnanolone. Moreover, the anxiolytic effects observed after progesterone administration were accompanied by increases in the sensitivity and efficacy of cortical $GABA_A$ receptors, as assessed by GABA-stimulated $^{36}Cl^-$ influx.

METHOD

Subjects and Experimental Protocol

Young adult, female rats (200–250 g) of the Long-Evans strain were purchased from Harlan-Sprague-Dawley (Altamont, NY) and maintained in a temperature-controlled colony room on a 12 L : 12 D cycle (light off at 1100 h) with free access to food and water. Two weeks after their arrival, females were ovariectomized using ketamine HCl (60 mg/kg, IP) and xylazine HCl (10 mg/kg, IP) as anesthetic agents. Following a 2-week recovery period, females were injected with 0, 1, or 4 mg progesterone (0.2 ml, SC) dissolved in a 37% cyclodextrin vehicle (Molecusol HPB). Four hours later (1400 h), ambulation in a locomotor activity chamber was monitored for 5 min, and immediately thereafter behavior in the elevated plus-maze was assessed for 5 min. After the behavioral tests, females were rapidly killed by cervical luxation and decapitated. Trunk blood was collected from a subgroup of females, centrifuged, and serum stored at −70°C for radioimmunoassay (RIA) of allopregnanolone. Brains were quickly removed and the cortex dissected on ice. Some cortical samples were stored at −70°C for assay of allopregnanolone by RIA, whereas the remaining cortical samples were freshly prepared for GABA-stimulated $^{36}Cl^-$ influx.

Locomotor Activity and Elevated Plus-maze Behavior

Ambulatory scores were determined in a Plexiglas arena (41 × 25 × 41 cm) placed in a sound attenuation chamber with an illumination intensity of 13–15 scotopic lux. Infrared photocells located along the walls of the test arena and separated by 5 cm from one another were connected to a cumulative recorder and provided an automated measure of locomotor activity. The elevated plus-maze consisted of two open arms (50 × 10 cm) and two enclosed arms (50 × 10 × 40 cm) with an open roof, arranged so that the two open arms were opposite one another. The maze was elevated to a height of 50 cm. Transparent Plexiglas rails (50 × 1 cm) were fixed to both sides of the open arms to prevent subjects from falling. Two lamps (25 W) were mounted 50 cm above the closed arms, providing a lighting intensity in the middle of the closed arms of 30 scotopic lux. The number of open and closed arm entries and the time spent in the open and closed arms were recorded by the experimenter. In the elevated plus-maze, anxiolytic effects are noted as an increase in the proportion of open arm entries and/or an increase in the proportion of time spent on the open arms relative to the total number of arm entries and total time spent on the open and closed arms; anxiogenic effects are noted as decreases in these measures (27).

RIA of Allopregnanolone

The RIA was performed on ether extracts of serum and cortical homogenates to which a recovery standard of ^{3}H-allopregnanolone (approximately 3,000 dpm) was added. Purification of allopregnanolone was achieved by high-performance liquid chromatography. Procedures involved in the endogenous steroid extraction, chromatography, and RIA have been published in detail elsewhere (31,32).

GABA-stimulated $^{36}Cl^-$ Influx

Cortical synaptoneurosomes were prepared and suspended in an assay buffer (20 mM HEPES-Tris, 118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, pH 7.4) at a concentration of 20 mg/ml, as described previously (4). Reaction vials were preincubated at 30°C for 15 min containing one of nine concentrations of GABA over a range of 1–1,000 μM and 0.5 μCi $^{36}Cl^-$ (New England Nuclear, Boston MA; specific activity = 16.48–17.59 mCi/g). A 100-μl aliquot of synaptoneurosomes (~2 mg protein) was added and the reaction was terminated 10 s later by addition of 5 ml cold buffer containing 100 μM picrotoxin and vacuum filtration. Cl^- uptake was measured in triplicate determinations using standard liquid scintillation spectrometry. Data are expressed as net Cl^- uptake in nmol/mg protein. Protein values were determined using a modified Lowry procedure (17). The maximal Cl^- transport and EC_{50} for GABA-stimulated Cl^- uptake were calculated using a computer-generated model (29).

Statistical Analyses

The parametric behavioral data were separately analyzed by a one-way analysis of variance (ANOVA), as were blood and brain allopregnanolone levels. Significant effects were further probed with posthoc linear comparisons. GABA-stimulated Cl^- influx data were analyzed by *t*-tests at each concentration of GABA. Maximal Cl^- uptake and EC_{50} for GABA-stimulated Cl^- influx were similarly analyzed by *t*-tests. Statistical significance was noted when the probability of a Type I error was < 0.05.

RESULTS

Progesterone injection in ovariectomized adult rats did not affect ambulation scores in a novel arena (Table 1). However, exploration of the elevated plus-maze was differentially affected by acute progesterone administration. ANOVA demonstrated that, whereas the total number of arm entries was not affected by progesterone injection, $F(2, 25) = 0.43$, a significant effect of progesterone was found for the number of open arm entries and time spent on the open arms, $F(2, 25) = 5.16, p < 0.01$, and $10.04, p < 0.001$, respectively. As shown in Table 1, an injection of 1 mg progesterone increased the time spent on the open arms but not the number of open arm entries. The 4-mg dose of progesterone increased both the

TABLE 1

AMBULATION IN AN AUTOMATED ACTIVITY CHAMBER AND EXPLORATION OF AN ELEVATED PLUS-MAZE IN OVARIECTOMIZED RATS 4 h AFTER ADMINISTRATION OF PROGESTERONE AT 0, 1, OR 4 mg (SC)

Progesterone	n	Activity	Number			Time		
			Total	Open	Closed	Total	Open	Closed
Vehicle	11	790.5 ± 20.7	10.5 ± 1.3	3.5 ± 0.8	7.0 ± 0.7	242.3 ± 10.0	38.1 ± 10.4	204.2 ± 13.9
1 mg	6	834.0 ± 39.6	11.8 ± 1.2	5.2 ± 0.9	6.7 ± 0.5	220.7 ± 16.5	72.0 ± 12.0*	148.7 ± 13.5
4 mg	11	729.6 ± 52.7	11.5 ± 0.4	6.1 ± 0.2†	5.4 ± 0.3	211.8 ± 8.3	95.1 ± 7.5†	116.7 ± 11.6

Data are expressed as mean ± SEM; activity scores are the number of photobeam interruptions per 5-min test session; time is reported in seconds. Significant effects were probed by posthoc linear comparisons against the vehicle group.

*$p < 0.05$.

†$p < 0.01$.

number of open arm entries and time spent on the open arms. A similar pattern of results is observed in an analysis of the ratios of the number of open arm entries to total number of arm entries and time spent on the open arms to the total time spent on the open and closed arms (Fig. 1). An ANOVA revealed a significant effect of progesterone administration on both of these measures, $F(2, 25) = 9.55$, $p < 0.001$, and 14.55, $p < 0.0001$, respectively. Posthoc linear comparisons showed that progesterone at 1 and 4 mg increased the proportion of time spent on the open arms whereas 4 mg progesterone also increased the proportion of number of open arm entries (Fig. 1). In this context, it is important to note that progesterone administration did not affect the total number of arm entries, further demonstrating the selective anxiolytic effect of progesterone administration (Table 1).

The effects of progesterone administration on blood serum and cortical level of allopregnanolone are summarized in Fig. 2A. A dose-dependent increase in serum and cortical allopregnanolone level was observed in progesterone-treated ovariectomized rats, $F(2, 6) = 14.06$, $p < 0.005$, and 52.84, $p < 0.0001$, respectively. As shown in Figs. 2B and 2C, the concentration of allopregnanolone in the cerebral cortex was significantly correlated to the proportion of open arm entries ($r = 0.59$, $p < 0.02$) and proportion of time spent on the open arms ($r = 0.68$, $p < 0.01$), respectively.

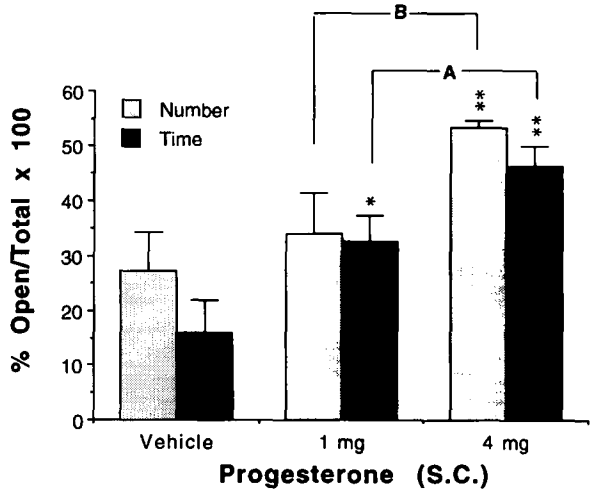

FIG. 1. Ratio of open arm entries to total number of arm entries and time spent on the open arms to total time spent on the open and closed arms. Data are mean ± SEM for ovariectomized rats given HPB vehicle ($n = 11$), 1 mg progesterone ($n = 6$), or 4 mg progesterone ($n = 11$) 4 h prior to behavioral testing. Posthoc linear comparisons with vehicle-treated group: *$p < 0.05$, **$p < 0.01$. Significant difference between progesterone at 1 mg and 4 mg: (A) $p < 0.05$; (B) $p < 0.01$.

GABA-stimulated $^{36}Cl^-$ transport in cortical synaptoneurosomes was enhanced in ovariectomized females treated with 4 mg progesterone relative to vehicle-treated ovariectomized rats. As shown in Fig. 3A, the concentration-dependent effect of GABA on Cl^- influx was increased in progesterone-treated animals at almost every concentration of GABA examined. Thus, 4 mg progesterone decreased the EC_{50}, $t(8) = 2.29$, $p < 0.05$ (Fig. 3B), and increased the maximal efficacy for GABA-stimulated Cl^- uptake, $t(8) = 2.94$, $p < 0.05$ (Fig. 3C). Each of these neurochemical measures were in turn significantly correlated to behavior in the elevated plus-maze, $r = -0.61$ and 0.68, $p < 0.05$, between the proportion of time spent on the open arms to EC_{50} and maximal efficacy, respectively.

DISCUSSION

The first goal of these studies was accomplished by the demonstration that progesterone administration elicited anxiolytic behavior and potentiated GABA-stimulated Cl^- influx in cortical synaptoneurosomes. Acute administration of progesterone to ovariectomized rats resulted in a dose-dependent increase in serum and cerebral cortical levels of allopregnanolone. Progesterone treatment also elicited significant anxiolytic behavior as measured in the elevated plus-maze. Brain allopregnanolone levels were positively correlated to exploratory behavior in the plus-maze. The effector response of the GABA$_A$ receptor in cortical tissue was increased in progesterone-treated females. Thus, the anxiolytic efficacy of progesterone treatment was accompanied by an increase in the potency (inverse of the EC_{50}) and an increase in the maximal efficacy of GABA$_A$ receptor-mediated Cl^- influx. Indeed, the effects of progesterone on the EC_{50} and maximal efficacy were each significantly correlated to the anxiolytic effect of progesterone on the elevated plus-maze. To our knowledge, these data are the first to demonstrate that the anxiolytic effects of progesterone are correlated to an increase in cortical allopregnanolone levels and an increase in cortical GABA$_A$ receptor function. These results further corroborate our previous findings that relative to ovariectomized rats GABA-stimulated Cl^- influx was increased in cortical synaptoneurosomes from female rats in proestrus (4), a stage of the estrous cycle characterized by peak circulating level of progesterone.

The second aim of these studies was to address the mechanisms mediating progesterone's putative effects. Steroid-induced genomic activation can alter neurochemistry and mor-

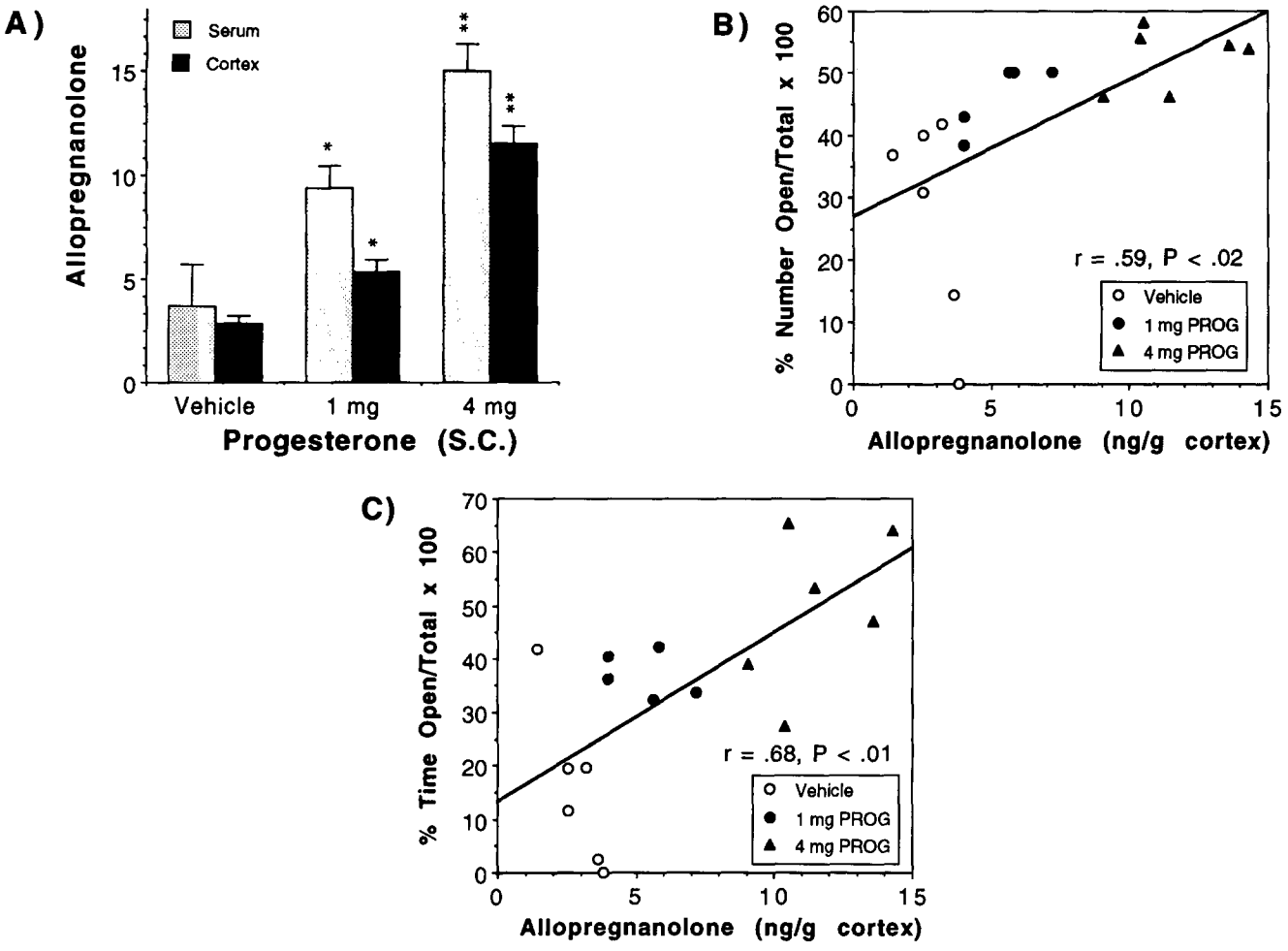

FIG. 2. (A) Serum (ng/ml) and cortical (ng/g) level of allopregnanolone 4 h after administration of progesterone (P: 0, 1, or 4 mg) in ovariectomized rats. Posthoc linear comparisons with vehicle-treated group: $*p < 0.05$, $**p < 0.005$. (B) Linear correlation between cortical allopregnanolone and proportion of open arm entries. (C) Linear correlation between cortical allopregnanolone and proportion of time spent on the open arms.

phology, thereby affecting neural function and behavior (19). A relatively novel concept, however, is that steroids may alter neural excitability via nongenomic effects mediated by membrane-bound receptors. The evidence favors the hypothesis that progesterone exerts its anxiolytic effects via its metabolism to allopregnanolone and that this metabolite subsequently augments $GABA_A$ receptor-mediated function in a nongenomic manner: a) The anxiolytic effects of progesterone were significantly correlated to the cortical levels of allopregnanolone; b) anxiolytic efficacy was also correlated to the maximal efficacy and potency of GABA-stimulated Cl^- transport; c) systemic and intracranial injection of allopregnanolone elicited anxiolytic activity that was blocked by coadministration of picrotoxin, a GABA-gated Cl^- blocker (3), but not by CGS-8216, a benzodiazepine receptor antagonist (41).

Whereas genomic effects are characterized by a long latency and duration, nongenomic effects occur within a short latency and are typically short-lived. However, this apparent dichotomy is not absolute. Latencies of steroid effects between minutes and hours may involve either nongenomic or genomic mechanisms. We chose to study behavioral and neurochemical indices of function of the $GABA_A$ receptor system 4 h after progesterone treatment to allow for adequate absorption and distribution of the steroid. The experimental protocol did not allow us to estimate the onset of progesterone's psychotropic and $GABA_A$ receptor agonist effects. In human female subjects, peak plasma allopregnanolone levels were achieved 2.6 h after progesterone administration, with a range of 1–6 h (Freeman and Purdy, personal communication). In the present studies, the temporal parameter within which progesterone's effects were observed is consistent with the notion that a lag period is necessary for the formation of the neuroactive metabolite from its parent hormone. It is important to note, however, that the anxiolytic effects of allopregnanol-one were observed within minutes of intracranial microinjection (3).

In experimental animals, the circulating levels of allopregnanolone parallel ovarian progesterone secretion (11,31); brain levels of allopregnanolone peak at proestrous, whereas low levels are found at diestrous (32). In addition to the production of allopregnanolone by the ovaries, CNS neurons and glia contain 5α-reductase and 3α-oxidoreductase activities, allowing for the in situ formation of allopregnanolone (12, 15,28). Indeed, significant levels of allopregnanolone were found in the brain of adrenalectomized and ovariectomized rats even in the absence of detectable levels of circulating allopregnanolone (32). It is notable, therefore, that in our

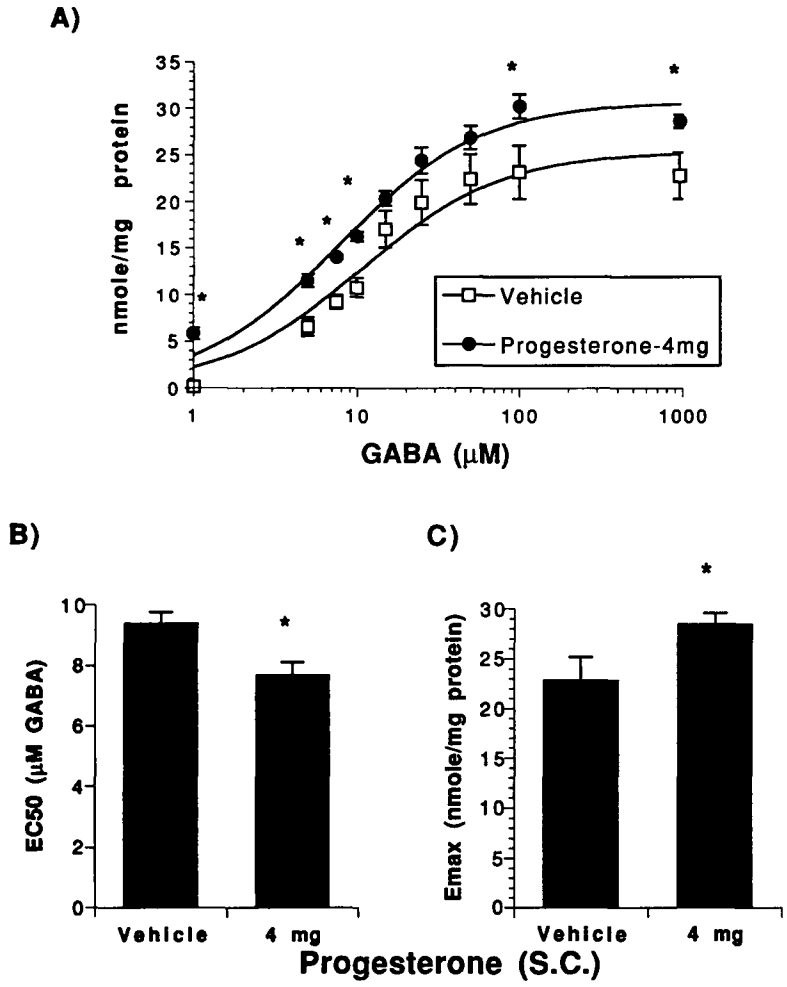

FIG. 3. (A) GABA-stimulated $^{36}Cl^-$ influx in cortical synaptoneurosomes from ovariectomized females administered vehicle (n = 5) or 4 mg progesterone (n = 5) 4 h prior to assay. (B) EC_{50} (μM GABA) and (C) maximal efficacy for GABA-stimulated Cl^- uptake. *Difference from vehicle at $p < 0.05$.

studies progesterone administration resulted in a dose-dependent accumulation of allopregnanolone in the brains of ovariectomized rats.

The physiological significance of our findings may be questioned because the effects were observed following injection of progesterone at pharmacological doses. We do not believe this to be the case, however, because the mean cortical level of allopregnanolone measured following 1 mg progesterone in our experiments was comparable to that previously reported in proestrous rats whereas that measured after 4 mg progesterone was comparable to that observed at gestational day 15 (26). Moreover, the level of cortical allopregnanolone associated with anxiolytic behavior in the present studies (3–6 ng/g or ng/ml, 10–20 nM) is comparable to concentrations observed to potentiate GABA-mediated responses in vitro (9, 23,30). That the high dose of progesterone may have affected other neurotransmitter systems through genomic-induced activation cannot be ruled out by these studies. However, further experiments are currently underway to determine the effects of progesterone administration on anxiolytic behavior and GABA$_A$ receptor-mediated Cl^- influx in the presence of a 5α-reductase inhibitor or a progestin receptor antagonist.

In summary, we reported that progesterone administration in ovariectomized rats produced anxiolytic behavior and increased cortical GABA$_A$ receptor function. These behavioral and neurochemical indices of function of the GABA$_A$ receptor were correlated with cortical levels of allopregnanolone. These studies corroborate recent clinical data regarding significant effects of progesterone on mood and cognitive or motor performance (8) and further substantiate the hypothesis that ovarian steroid hormone metabolites may play a role as endogenous modulators of the GABA$_A$ receptor. It is anticipated that further research concerning the mechanism of action underlying progesterone's psychotropic effects may provide insight into the suspected neuroendocrinological component of psychiatric conditions of premenstrual anxiety and postpartum depression.

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