

# Allopregnanolone Synthesis in Cerebellar Granule Cells: Roles in Regulation of GABA<sub>A</sub> Receptor Expression and Function during Progesterone Treatment and Withdrawal

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## ABSTRACT

Rat cerebellar granule cells were cultured for 5 days with progesterone, resulting in the conversion of progesterone to allopregnanolone, a potent and efficacious modulator of  $\gamma$ -aminobutyric acid (GABA) type-A receptors, as well as in decreases in the abundance of GABA<sub>A</sub> receptor  $\alpha_1$ ,  $\alpha_3$ ,  $\alpha_5$ , and  $\gamma_2$  subunit mRNAs. These effects were accompanied by decreases in the efficacies of diazepam and the  $\beta$ -carboline DMCM with regard to modulation of GABA-evoked Cl<sup>-</sup> currents. Withdrawal from such progesterone treatment resulted in

a rapid and selective increase in the abundance of the GABA<sub>A</sub>  $\alpha_4$  subunit mRNA that was associated with a restoration of receptor sensitivity to the negative modulatory action of DMCM, a positive receptor response to flumazenil, and continued reduced responsiveness of receptors to diazepam. Prevention of allopregnanolone synthesis by the  $5\alpha$ -reductase inhibitor finasteride also prevented the changes in both GABA<sub>A</sub> receptor gene expression and receptor function elicited by progesterone treatment and withdrawal.

The discovery that modulation of  $\gamma$ -aminobutyric acid (GABA) type-A receptors by various psychoactive drugs underlies the pharmacological activity of these agents stimulated the search for endogenous molecules that are able to interact with the allosteric recognition sites associated with the receptor complex. Certain endogenous steroids were thus subsequently shown to modulate GABAergic transmission with potencies and efficacies similar to or greater than those of benzodiazepines or barbiturates (Harrison and Simmonds, 1984; Majewska et al., 1986). Evidence that such steroids are synthesized de novo from cholesterol in the central nervous system (CNS) (Le Goascogne et al., 1987; Mellon and Deshepper, 1993; Prasad et al., 1994; Baulieu, 1998) further indicated that "neurosteroids" might function as selective endogenous modulators of central GABA<sub>A</sub> receptor-mediated neurotransmission.

More recently, physiological and pharmacologically induced fluctuations in the plasma or brain concentrations of allopregnanolone (AP), a  $5\alpha$ -reduced,  $3\alpha$ -hydroxylated metabolite of progesterone, have been shown to modulate GABA<sub>A</sub> receptor plasticity and associated behavior (Weiland and Orchinik, 1995; Concas et al., 1998; Follesa et al., 1998;

Smith et al., 1998a,b). A number of studies demonstrate the effect of chronic steroid exposure on the GABA<sub>A</sub> receptor pharmacology in cultured neurons (Yu and Ticku, 1995; Friedman et al., 1996). These various observations have suggested that changes in the production of neurosteroids, and consequent changes in the brain concentration of AP, might contribute directly not only to normal physiology but also to a variety of neurological and psychiatric disorders that are characterized by changes in emotional state, sleep pattern, and neuronal excitability. Indeed, a selective decrease in the plasma and cerebrospinal fluid concentrations of AP, as well as normalization of these concentrations after treatment, has been described in individuals with major depression (Ströhle et al., 1999).

To characterize further the contributions to GABA<sub>A</sub> receptor modulation of both neurosteroids synthesized de novo in the CNS and those produced in the CNS from precursors such as progesterone synthesized in the periphery, we have now studied cultures of rat cerebellar granule cells. These cultures contain ~95% granule cells and 5% other cell types, including glial cells. These neurons are mainly glutamatergic, and they express all the 14 mRNA subunits of the GABA<sub>A</sub> receptor (Bovolin et al., 1992) differently from the cerebellum in the adult rat, where only discrete  $\alpha$  subunits are present (Laurie et al., 1992). We investigated the ability

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of these cultured neurons to produce 5 $\alpha$ -reduced, 3 $\alpha$ -hydroxylated metabolites of progesterone. Although various brain regions express enzymes required for neurosteroid synthesis, the cellular localization of most of these enzymes is not known (Mensah-Nyagan et al., 1999). Indeed, it remains unclear whether de novo synthesis of neurosteroids occurs only in oligodendrocytes and astrocytes or also in neurons and other glial cell types. Thus, we first determined whether cerebellar granule cell cultures express 5 $\alpha$ -reductase, which is required for the conversion of progesterone to AP, and whether this enzyme is localized to the neurons or glial cells.

We next investigated whether progesterone is converted to AP by the cultured cells, and whether this metabolite exerts a tonic effect on the GABA<sub>A</sub> receptor complex. In addition, we evaluated whether long-term exposure of cerebellar granule cells to the endogenously produced neurosteroid differentially modulates the expression of GABA<sub>A</sub> receptor subunit genes in a manner similar to that apparent in the brain of pregnant rats (Fenelon and Herbison, 1996; Brussaard et al., 1997; Concas et al., 1998; Follesa et al., 1998). Moreover, the expression of GABA<sub>A</sub> receptor subunit genes was also examined after progesterone withdrawal, with particular regard to the expression of the  $\alpha_4$  subunit gene. The expression of the  $\alpha_4$  subunit, which affects the sensitivity of GABA<sub>A</sub> receptors to both positive and negative allosteric modulators (for review, see Barnard et al., 1998), has been shown to be increased in the brain of rats during progesterone withdrawal (Smith et al., 1998a,b). Finally, to evaluate whether progesterone-induced changes in GABA<sub>A</sub> receptor gene expression were associated with alterations in receptor function, we transplanted native GABA<sub>A</sub> receptors from cultured granule cells that had been subjected to long-term treatment with progesterone or to progesterone withdrawal into *Xenopus* oocytes and, with the use of the voltage-clamp technique, measured their pharmacological responses to various GABA<sub>A</sub> receptor modulators.

## Materials and Methods

**Cell Culture.** Primary cultures of cerebellar neurons enriched in granule cells were prepared from cerebella of 8-day-old rats (Bovolín et al., 1992). These cells in culture for 8 days express all the 14 subunit mRNAs of the GABA<sub>A</sub> receptor (Bovolín et al., 1992) with an expression pattern similar to that observed in the postnatal developing cerebellum but different from that observed in the adult rat cerebellum (Laurie et al., 1992). Cells were plated ( $2.5 \times 10^6$  cells/2 ml) in 100-mm dishes that had been coated with poly-L-lysine (10  $\mu$ g/ml; Sigma, St. Louis, MO) and were cultured in basal Eagle's medium (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 2 mM glutamine, gentamicin (100  $\mu$ g/ml; Sigma), and 25 mM KCl. This high concentration of potassium was necessary to have a chronic depolarization condition and promote the survival of granule cells. Cytosine arabinofuranoside (1  $\mu$ M final concentration; Sigma) was added to cultures 18 h after plating to inhibit the proliferation of non-neuronal cells. Cells were maintained for a total of 8 days in culture, so that chronic treatment with progesterone was initiated accordingly. Progesterone, AP, and finasteride were dissolved in dimethyl sulfoxide and diluted sequentially in culture medium to have a final concentration of 1  $\mu$ M; control cells were treated with solvent alone at the same dilution as the one present in the drug-treated cells. The culture medium was replaced every day with fresh medium containing the indicated drugs.

**Probe Preparation.** Total RNA was extracted from rat brain (Follesa et al., 1998) and subjected to reverse transcription with SuperScript reverse transcriptase (Life Technologies) in the presence of oligo(dT). The resulting cDNA (1 to 10 ng) was amplified by the polymerase chain reaction (Follesa et al., 1998) with *Taq* DNA polymerase (2.5 U) (Perkin-Elmer/Cetus, Norwalk, CT) in 100  $\mu$ l of standard buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl<sub>2</sub>, 0.01% gelatin] containing 1  $\mu$ M each of specific sense and antisense primers and 200 mM deoxynucleoside triphosphate. The primer pairs for the different subunits of the GABA<sub>A</sub> receptor (Follesa et al., 1998) were designed to include cDNA sequences with the lowest degree of homology among the different receptor subunits (Follesa et al., 1998). The sense and antisense primers used for 5 $\alpha$ -reductase type I were 5'-CCT GGC CGC TGT ACG AGT ACA TTC-3' and 5'-GCC ACA CCA CTC CAC GAG CTC CCC-3', respectively (Anderson et al., 1989). The reaction was performed in a thermal cycler (Ericomp, San Diego, CA) for 30 cycles of 94°C for 45 s, 60°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 15 min (Follesa et al., 1998). The reaction products were separated by electrophoresis on a 1.8% low-melting-point agarose gel, visualized by staining with ethidium bromide, excised from the gel, purified, and inserted into the pAMP 1 cloning vector (Life Technologies). *Escherichia coli* NM522 was transformed with the resulting plasmids, which were subsequently purified from the bacteria, and the cDNA inserts were sequenced with a Sequenase DNA sequencing kit (USB, Cleveland, OH). The determined nucleotide sequences were 100% identical with the respective previously published sequences.

Plasmids containing the cDNA fragments corresponding to the various GABA<sub>A</sub> receptor subunits were linearized with restriction enzymes (Follesa et al., 1998). The plasmid containing the cDNA fragment corresponding to steroid 5 $\alpha$ -reductase (nucleotides 191 to 621) was linearized with *Hind*III. Linearized plasmids were used as a template, together with the appropriate RNA polymerase (SP6 or T7), to generate [ $\alpha$ -<sup>32</sup>P]CTP-labeled cRNA probes for RNase protection assays.

**RNase Protection Assay.** An RNase protection assay was used as a sensitive technique for semiquantitative detection of RNA and was performed as previously described (Follesa et al., 1998). This assay was used to measure the abundance of the  $\alpha_1$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_5$ ,  $\beta_1$ ,  $\beta_2$ , and the two splicing variant of the  $\gamma_2$  subunit, the  $\gamma_{2L}$  and  $\gamma_{2S}$  mRNAs or the mRNA encoding the 5 $\alpha$ -reductase. Total RNA was extracted from cultured cerebellar granule cells and quantified by measurement of absorbance at 260 nm. Briefly, 25  $\mu$ g of total RNA was dissolved in 20  $\mu$ l of hybridization solution containing 150,000 cpm of <sup>32</sup>P-labeled cRNA probe for a specific GABA<sub>A</sub> receptor subunit or 5 $\alpha$ -reductase (specific activity,  $6 \times 10^7$  to  $7 \times 10^7$  cpm/ $\mu$ g) and 15,000 cpm of <sup>32</sup>P-labeled cyclophilin cRNA (specific activity,  $1 \times 10^6$  cpm/ $\mu$ g). Cyclophilin is expressed widely among tissues, including the brain, and its gene is most likely regulated in an "on or off" manner (Follesa et al., 1998); cyclophilin mRNA was thus used as an internal standard for our measurements. The hybridization reaction mixtures were incubated at 50°C overnight and then subjected to digestion with RNase, after which RNA-RNA hybrids were detected by electrophoresis (on a sequencing gel containing 5% polyacrylamide and urea) and autoradiography. The amounts of GABA<sub>A</sub> receptor subunit mRNAs and cyclophilin mRNA were determined by measuring the optical density of the corresponding bands on the autoradiogram with a densitometer (model GS-700; Bio-Rad, Hercules, CA); this instrument is calibrated to detect saturated values, so that all our measurements were in the linear range. The data were normalized by dividing the optical density of the protected fragment for each receptor subunit mRNA by that of the respective protected fragment for cyclophilin mRNA. The amount of mRNA was therefore expressed in arbitrary units.

**Steroid Extraction and AP Radioimmunoassay.** Steroids were extracted and purified as previously described (Purdy et al., 1990). Briefly, steroids present in cerebellar granule cell homoge-

nates (prepared in 3 ml of PBS, pH 7.0) or in culture medium (10 ml, freeze-dried) were extracted three times with ethyl acetate, and the combined organic phases were dried under vacuum. The resulting residue was dissolved in 4 ml of *n*-hexane and applied to a SepPak silica cartridge, and components were eluted with *n*-hexane and 2-propanol (7:3, v/v). Steroids were separated and further purified by high-performance liquid chromatography on a 5- $\mu$ m Lichrosorb-diol column (250  $\times$  4 mm) with a discontinuous gradient of 2-propanol (0–30%) in *n*-hexane. The recovery (70–80%) of AP through the extraction and purification procedures was monitored by adding a trace amount (6000–8000 cpm; 20–80 Ci/mmol) of tritiated standard to the samples. AP was quantified by radioimmunoassay as previously described (Purdy et al., 1990; Concas et al., 1998) with a specific antibody generated in sheep, and characterized as previously described (Purdy et al., 1990).

**In Situ Hybridization.** Cells grown on coverslips for 8 days were fixed in 4% paraformaldehyde and subjected to in situ hybridization with the use of an Amersham RNA color kit (Amersham Life Science, Amersham, UK). The cRNA probe for 5 $\alpha$ -reductase was the same as that used for RNase protection assays, with the exception that it was labeled with fluorescein-11-UTP instead of [ $\alpha$ - $^{32}$ P]CTP. The presence of 5 $\alpha$ -reductase mRNA was detected as a dark blue-purple precipitate.

**Preparation of Membranes from Cultured Granule Cells and Their Injection into Oocytes.** After aspiration of culture medium, granule cells were carefully washed twice with 3-ml portions of an ice-cold solution containing 10 mM HEPES-NaOH (pH 7.5) and 0.1 mM EDTA. The cells were then scraped into 3 ml of the same solution and homogenized on ice with a Teflon pestle and glass homogenizer (six to eight strokes). The homogenate was centrifuged at 48,000g for 15 min at 4°C, and the resulting pellet was resuspended in 5 ml of ice-cold homogenization buffer and centrifuged again at 48,000g for 15 min. The final pellet was resuspended in the same buffer, adjusted to a protein concentration of 2 to 4 mg/ml, divided into aliquots, and stored at –20°C until use.

Stage V and VI oocytes were isolated from adult *Xenopus laevis* females (Dipl. Biol.-Dipl. Ing. Horst Kähler, Hamburg, Germany) and exposed to collagenase type IA as described previously (Sanna et al., 1998). A portion (50–100 nl) of granule cell membrane suspension (2–4 mg/ml) was injected into the cytoplasm of each oocyte. Injected oocytes were cultured until use in modified Barth's saline [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO<sub>3</sub>, 10 mM HEPES-NaOH (pH 7.5), 0.82 mM MgSO<sub>4</sub>, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.91 mM CaCl<sub>2</sub>] supplemented with 2 mM sodium pyruvate, penicillin (10 U/ml), streptomycin (10 mU/ml), gentamicin (50 mU/ml), and 0.5 mM theophylline. Oocytes were usually maintained for up to 3 days, during which time they were transferred to fresh incubation medium each day.

**Electrophysiological Recording.** Electrophysiological recording was initiated 12 to 18 h after injection of oocytes with granule cell membranes. The oocytes were placed in a rectangular recording chamber (volume, 100  $\mu$ l) and continuously perfused with modified Barth's saline at a flow rate of 2 ml/min and room temperature. They were impaled at the animal pole with two microelectrodes (resistance, 0.3 to 3 M $\Omega$ ) filled with filtered 3 M KCl and were subjected to voltage clamp at –90 mV with an Axoclamp 2-B amplifier (Axon Instruments, Burlingame, CA). The resting membrane potential usually ranged between –30 and –50 mV. Drugs were applied for 20 s; intervals of 5 min were allowed between applications of low concentrations of GABA alone and of  $\geq 10$  min when GABA was applied at higher concentrations or with other drugs. When testing the effects of various modulators on GABA-evoked currents, a GABA concentration that produced  $10 \pm 2\%$  (EC<sub>10</sub>) of the maximal current amplitude evoked by 10 mM GABA was used as a control response; this concentration was experimentally determined for each oocyte at the beginning of the recording.

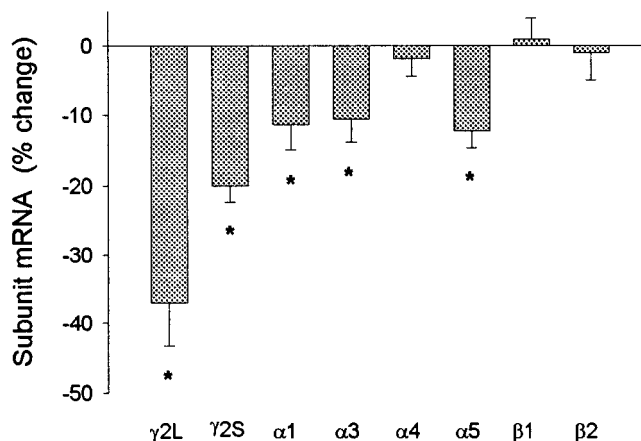
**Statistical Analysis.** Data are presented as means  $\pm$  S.E. The statistical significance of differences was assessed by analysis of

variance followed by Scheffe's test. A *P* value of  $<.05$  was considered statistically significant.

## Results

**Effects of Long-Term Exposure to Progesterone on GABA<sub>A</sub> Receptor Gene Expression.** RNase protection assays revealed that exposure of cultures of cerebellar granule cells to 1  $\mu$ M progesterone for 5 days resulted in a marked decrease in the abundance of transcripts encoding the  $\gamma_2$  subunit of GABA<sub>A</sub> receptors (Fig. 1). Similar treatment of these cells with 1  $\mu$ M AP reduced the abundance of both  $\gamma_{2S}$  and  $\gamma_{2L}$  subunit transcripts by  $27 \pm 3$  and  $40 \pm 8\%$ , respectively ( $P < .01$ ). The effect of progesterone on the amount of  $\gamma_{2L}$  subunit mRNA (–37%) was greater than that on the amount of  $\gamma_{2S}$  subunit mRNA (–20%). Long-term exposure of cultures to progesterone also significantly reduced the abundance of mRNAs encoding the  $\alpha_1$ ,  $\alpha_3$ , and  $\alpha_5$  receptor subunits, but it had no effect on that of the  $\alpha_4$ ,  $\beta_1$ , or  $\beta_2$  subunit mRNAs (Fig. 1). The effect of progesterone on the amounts of  $\alpha_1$ ,  $\alpha_3$ , and  $\alpha_5$  subunit mRNAs ( $\sim 13\%$ ) was less marked than that on the abundance of  $\gamma_2$  transcripts.

**AP Synthesis by Cerebellar Granule Cells.** Given that progesterone itself does not exert a direct modulatory effect on GABA<sub>A</sub> receptor function at 1  $\mu$ M concentration (Majewska et al., 1986; Wu et al., 1990), we investigated whether the effects of long-term treatment of cultured cerebellar granule cells with this steroid on the abundance of GABA<sub>A</sub> receptor subunit mRNAs were due to a direct action of progesterone itself or to the action of its metabolite AP at the GABA<sub>A</sub> receptor. We first examined whether the cerebellar granule cell cultures synthesize 5 $\alpha$ -reductase mRNA. RNase protection assay detected 5 $\alpha$ -reductase transcripts in the cultured cells (Fig. 2A). Furthermore, in situ hybridization revealed the presence of 5 $\alpha$ -reductase mRNA within the cell bodies of cerebellar granule cells (Fig. 2B). The abundance of 5 $\alpha$ -reductase mRNA was not affected by exposure of the cultures to 1  $\mu$ M progesterone for 5 days [control ( $100 \pm 5\%$ ,  $n = 9$ ), progesterone ( $107 \pm 5\%$ ,  $n = 14$ );  $P = .33$ ] (Fig. 2A). Our cell



**Fig. 1.** Effects of long-term exposure of cerebellar granule cell cultures to progesterone on the abundance of GABA<sub>A</sub> receptor subunit mRNAs. Cultured cerebellar granule cells were incubated for 5 days in the presence of 1  $\mu$ M progesterone, after which total RNA was extracted and the amounts of receptor subunit mRNAs were measured by RNase protection assay. Data are expressed as the percentage of change in transcript abundance compared with values for control (vehicle-treated) cells, and are means  $\pm$  S.E. ( $n = 13$  to 23) of values from three to four independent experiments. \* $P < .01$  versus control cells.



culture preparations also expressed low levels of the 3 $\alpha$ -hydroxysteroid oxidoreductase as revealed by RNase protection assay (data not shown). Consistent with the presence of 5 $\alpha$ -reductase mRNA in the cerebellar granule cells, we detected high concentrations of AP in both the cells and conditioned medium of cultures incubated in the presence of progesterone (Table 1). Thus, exposure of cultures to 1  $\mu$ M progesterone for 5 days resulted in 5- and 3-fold increases in the amounts of AP in the cells and medium, respectively. This conversion of progesterone to AP by the cultured cells was blocked (Table 1) by the 5 $\alpha$ -reductase inhibitor finasteride (Rittmaster, 1994). The notion that the effects of progesterone on the abundance of GABA<sub>A</sub> receptor subunit mRNAs was mediated by endogenously synthesized AP was supported by the observation that finasteride also blocked the progesterone-induced decrease in the amounts of  $\gamma_{2L}$  and  $\gamma_{2S}$  transcripts in the granule cell cultures (Fig. 3). Finasteride alone had no effect on  $\gamma_2$  transcript abundance. The presence or absence of glial cells in the cerebellar granule cell cultures did not appear to affect either the progesterone-

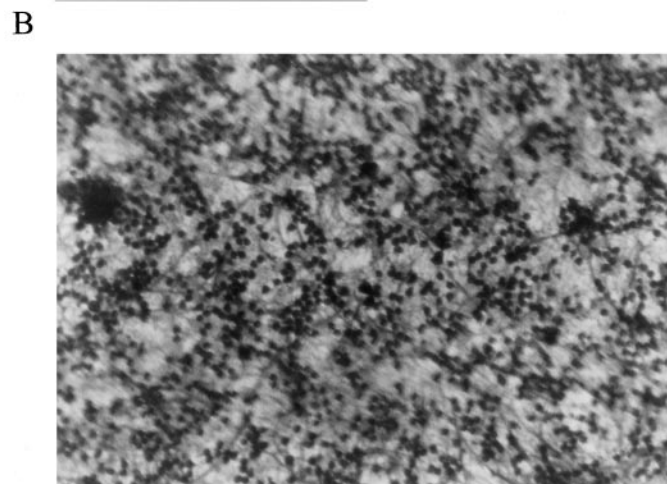
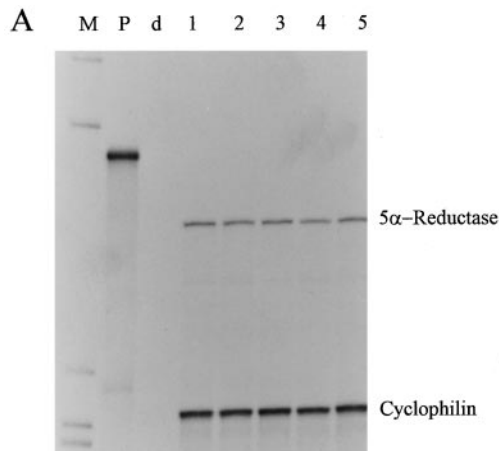
induced decrease in the abundance of the  $\gamma_{2L}$  subunit mRNA or the increase in AP concentration (data not shown).

**Effects of Progesterone Withdrawal on GABA<sub>A</sub> Receptor Gene Expression.** We next investigated the effects of progesterone withdrawal on the abundance of GABA<sub>A</sub> receptor subunit mRNAs in cultured cerebellar granule cells. Withdrawal of progesterone after exposure to this steroid for 5 days resulted in marked, time-dependent changes in the abundance of the  $\alpha_4$  subunit mRNA (Fig. 4). The amount of  $\alpha_4$  mRNA first increased, reaching a maximum (+25%) 6 h after progesterone withdrawal; decreased below control levels, reaching a minimum (−30%) at 12 and 24 h after withdrawal; and finally returned to control values 48 h after progesterone removal. The abundance of the mRNAs encoding  $\alpha_1$  and  $\gamma_{2L}$  subunits remained significantly decreased (relative to control values) 6 h after progesterone withdrawal (Fig. 5); after incubation of cells in the absence of progesterone for an additional 18 h, the amounts of these mRNAs did not differ significantly from control values. For cells incubated for 5 days with both progesterone and finasteride, the amounts of  $\alpha_1$  and  $\alpha_4$  mRNAs measured 6 h after withdrawal of both drugs did not differ significantly from control values (Fig. 6).

**Effects of Chronic Progesterone Treatment and Progesterone Withdrawal on GABA<sub>A</sub> Receptor Function.** To investigate whether the changes in GABA<sub>A</sub> receptor gene expression induced in cerebellar granule cells by long-term exposure to progesterone and by progesterone withdrawal are accompanied by changes in GABA<sub>A</sub> receptor function, we transplanted GABA<sub>A</sub> receptors from cultured granule cells to *Xenopus* oocytes and characterized their functional properties with the voltage-clamp technique. In fact, the transplanted receptors are efficiently inserted into the oocyte plasma membrane where they form “clusters” of receptors that retain their native properties (Morales et al., 1995; Sanna et al., 1998). Receptor transplantation was accomplished by injecting crude membrane vesicles prepared from granule cells into the oocytes. We have previously shown (Sanna et al., 1998) that this procedure leads to the incorporation of preformed GABA<sub>A</sub> receptors into the oocyte membrane, likely as a result of fusion of the injected membrane vesicles with the oocyte membrane. Twelve to 18 h after injection of oocytes with granule cell membrane vesicles, GABA induced an inward Cl<sup>−</sup> current with a peak amplitude that was dependent on the concentration of the neurotransmitter; maximal current amplitudes, induced by 10 mM GABA, usually ranged from 100 to 200 nA.

The benzodiazepine diazepam markedly potentiated GABA-evoked Cl<sup>−</sup> currents in oocytes expressing GABA<sub>A</sub> receptors from control granule cells (Fig. 7A). This effect was concentration-dependent, with potentiation values of  $74.4 \pm 12$  and  $102.6 \pm 4\%$  at 1 and 3  $\mu$ M diazepam, respectively. In oocytes injected with membrane vesicles prepared from granule cells after exposure to progesterone for 5 days, the potentiating effect of diazepam was much less pronounced ( $27.5 \pm 2$  and  $31.6 \pm 2\%$  at 1 and 3  $\mu$ M, respectively). Similarly, in oocytes expressing GABA<sub>A</sub> receptors transplanted from granule cells 6 h after progesterone withdrawal, diazepam potentiated GABA-evoked Cl<sup>−</sup> currents by only  $23.9 \pm 6$  and  $27.0 \pm 9\%$  at 1 and 3  $\mu$ M, respectively.

The benzodiazepine receptor antagonist flumazenil (1  $\mu$ M) had no significant effect on GABA-evoked Cl<sup>−</sup> currents in



**Fig. 2.** Expression of the 5 $\alpha$ -reductase gene in cerebellar granule cells. A, RNase protection assay of 5 $\alpha$ -reductase mRNA. Cultured cerebellar granule cells were incubated for 5 days in the presence (lanes 1 to 3) or absence (lanes 4 and 5) of 1  $\mu$ M progesterone, after which total RNA was extracted and subjected to RNase protection assay of 5 $\alpha$ -reductase and cyclophilin (control) mRNAs. Lane M, molecular size markers  $\phi$ X174 HaeIII-digested; lane P, 5 $\alpha$ -reductase and cyclophilin probes alone; and lane d, digested 5 $\alpha$ -reductase and cyclophilin probes. B, in situ hybridization of cultured cerebellar granule cells with a 5 $\alpha$ -reductase cRNA probe. The presence of 5 $\alpha$ -reductase mRNA in the cerebellar granule cells is revealed by the dark precipitate.

oocytes injected with membranes from either control granule cells or those subjected to long-term progesterone treatment (Fig. 7B). In contrast, flumazenil increased GABA-evoked  $\text{Cl}^-$  currents by  $44.4 \pm 6\%$  in oocytes expressing GABA<sub>A</sub> receptors transplanted from granule cells 6 h after progesterone withdrawal. The anxiogenic and convulsant  $\beta$ -carboline derivative DMCM, a benzodiazepine receptor inverse agonist, induced a marked inhibition ( $34.1 \pm 8$  and  $40.5 \pm 9\%$  at 0.3 and 1  $\mu\text{M}$ , respectively) of GABA-evoked  $\text{Cl}^-$  currents in oocytes injected with membranes from control granule cells (Fig. 7C). Consistent with previous results (Whittemore et al., 1996), higher concentrations (10 to 30  $\mu\text{M}$ ) of this drug enhanced the response of control GABA<sub>A</sub> receptors to GABA (data not shown). In oocytes expressing GABA<sub>A</sub> receptors from progesterone-treated granule cells, DMCM at 0.3 or 1  $\mu\text{M}$  had no effect on GABA-evoked  $\text{Cl}^-$  currents. However, this drug inhibited the GABA response by  $32.3 \pm 6$  and  $35.3 \pm 8\%$  at 0.3 and 1  $\mu\text{M}$ , respectively, in oocytes expressing GABA<sub>A</sub> receptors transplanted from granule cells 6 h after progesterone withdrawal. The inhibitory effect of DMCM on GABA-evoked  $\text{Cl}^-$  currents in oocytes injected with membrane vesicles from either control granule cells or those subjected to progesterone withdrawal was completely blocked by 1  $\mu\text{M}$  flumazenil. Finally, the changes in diazepam, flumazenil, and DMCM sensitivity apparent with GABA<sub>A</sub> receptors from both progesterone-treated granule cells and those subjected to progesterone withdrawal were prevented by the inclusion of finasteride in the 5-day incubation of granule cells with progesterone (data not shown).

## Discussion

We have now shown that long-term exposure of cultured cerebellar granule cells to progesterone mimics the effects of chronic treatment of these cells with high concentrations of AP as well as the effects of pregnancy (Fenelon and Herbison, 1996; Brussaard et al., 1997; Concas et al., 1998; Follesa et al., 1998) on the expression of specific GABA<sub>A</sub> receptor subunit genes. Given that AP, but not progesterone (1  $\mu\text{M}$ ) exhibits a positive allosteric modulatory action at GABA<sub>A</sub> receptors, our data suggest that AP, produced as a result of progesterone metabolism by neurons, is responsible for the observed progesterone-induced changes in GABA<sub>A</sub> receptor gene expression via a nongenomic mechanism as previously suggested (Concas et al., 1998).

Consistent with this notion, we detected 5 $\alpha$ -reductase mRNA in the cerebellar granule cells and showed that the

addition of progesterone to the primary cultures resulted in marked increases in AP concentration. These data are also consistent with those of previous biochemical studies showing that both primary cultures of neurons (Melcangi et al., 1994), astrocytes, and oligodendrocytes (Tsuruo et al., 1996), express the 5 $\alpha$ -reductase. Thus, our data suggest that, in this primary culture system, cerebellar granule cells are the major source of AP.

Our results on the effect of chronic progesterone modulating the gene expression of the GABA<sub>A</sub> receptor are supported by previous *in vivo* studies (Fenelon and Herbison, 1996; Brussaard et al., 1997; Concas et al., 1998; Smith et al., 1998a,b). Moreover, our data are consistent with the observation that chronic treatment with agonists or positive allosteric modulators of the GABA<sub>A</sub> receptor results in down-regulation of the receptor by decreasing the abundance of specific receptor subunit mRNAs (Roca et al., 1989; Morrow et al., 1990; Montpied et al., 1991; Impagnatiello et al., 1996; Yu et al., 1996). Furthermore, we showed that long-term exposure of granule cells to progesterone resulted in a marked decrease in the ability of diazepam to potentiate GABA-evoked  $\text{Cl}^-$  currents, consistent with the reduced abundance of  $\alpha_1$ ,  $\alpha_3$ ,  $\alpha_5$ , and  $\gamma_2$  subunit mRNAs. Both  $\alpha$  and  $\gamma_2$  subunits are required for GABA<sub>A</sub> receptors to show maximal sensitivity to benzodiazepines as well as to benzodiazepine receptor inverse agonists (Pritchett et al., 1989; Barnard et al., 1998). Because the exact relationship between receptor subunit mRNA and proteins expressed on the cell surface is unknown, an alteration in the mRNA levels cannot be interpreted as changes in GABA<sub>A</sub> receptor subunit gene expression, but a change in synthesis that could consequently produce a change in receptor expression, and in turn, lead to potential changes in receptor function. The mechanisms under which changes in GABA<sub>A</sub> receptor synthesis and function can occur could be either post-transcriptional (e.g., mRNA stability) and/or post-translational (e.g., phosphorylation, receptor assembly). This last possibility has been also described in other systems *in vitro* (Klein et al., 1996).

Chronic progesterone treatment also reduced the inhibitory effect of low concentrations (0.3 and 1  $\mu\text{M}$ ) of the  $\beta$ -carboline DMCM, an anxiogenic and convulsant drug that acts at the benzodiazepine receptor (Biggio et al., 1995). As shown in previous studies (Whittemore et al., 1996), we found that higher concentrations (10 to 30  $\mu\text{M}$ ) of DMCM potentiated the GABA response; however, because this effect is not sensitive to flumazenil, it is likely mediated through a different,

TABLE 1  
Effects of progesterone and finasteride on the amount of AP in cells and conditioned medium of cerebellar granule cell cultures

Treatment	AP			
	Cells		Medium	
	% of control	ng/g protein	% of control	ng/ml medium
Control	100 $\pm$ 9	0.60 $\pm$ 0.05	100 $\pm$ 5	0.56 $\pm$ 0.03
Progesterone	563 $\pm$ 123 <sup>a</sup>	3.40 $\pm$ 0.70	269 $\pm$ 34 <sup>a</sup>	1.50 $\pm$ 0.19
Finasteride + progesterone	106 $\pm$ 12 <sup>b</sup>	0.63 $\pm$ 0.07	102 $\pm$ 14 <sup>b</sup>	0.57 $\pm$ 0.08
Finasteride	70 $\pm$ 5	0.42 $\pm$ 0.03	68 $\pm$ 7	0.38 $\pm$ 0.04

Cultures were incubated for 5 days in the absence or presence of 1  $\mu\text{M}$  progesterone or 1  $\mu\text{M}$  finasteride as indicated, after which the amount of AP in cells and medium was measured by radioimmunoassay. Data are expressed as the percentage of control (solvent), and are means  $\pm$  S.E. of values from four independent experiments ( $n = 11$  to 16).

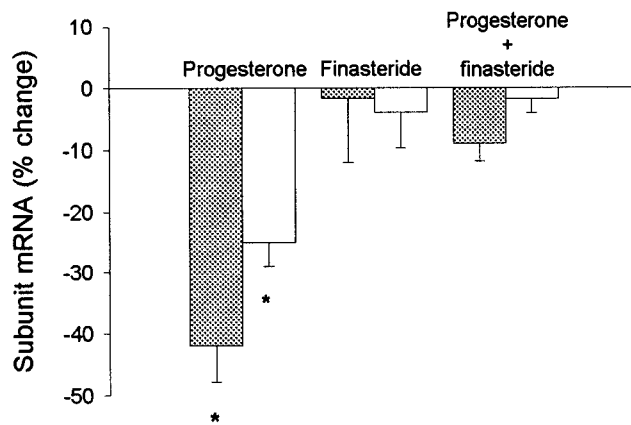
<sup>a</sup>  $P < .01$  versus solvent.

<sup>b</sup>  $P < .01$  versus progesterone.

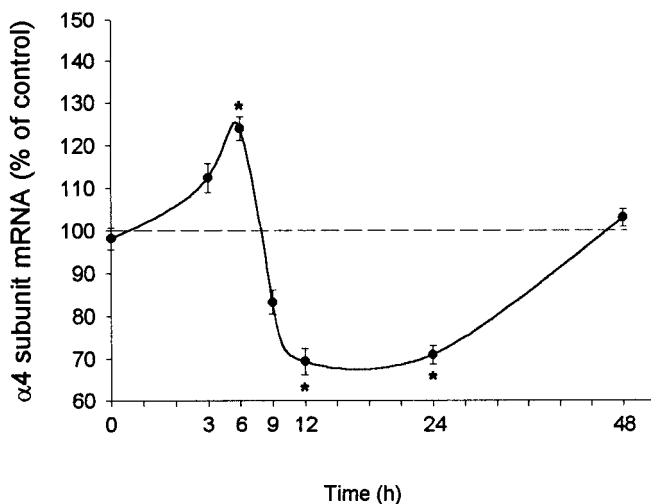
low-affinity site that is not modulated as a result of long-term exposure to progesterone.

Our observation that finasteride, a potent inhibitor of 5 $\alpha$ -reductase (Rittmaster, 1994), prevented both the conversion of progesterone to AP as well as the effect of progesterone on the abundance of GABA<sub>A</sub> receptor subunit mRNAs in granule cell cultures provides direct evidence that progesterone metabolites produced by these neurons modulates GABA<sub>A</sub> receptor plasticity. AP is thought to play a similar role during pregnancy and pseudopregnancy in rats (Concas et al., 1998; Follesa et al., 1998; Smith et al., 1998a,b). The ability of granule cells to metabolize progesterone to AP further established the idea (Paul and Purdy, 1992; for review see Baulieu, 1998) that, in vivo, the neuronal metabolism of progesterone

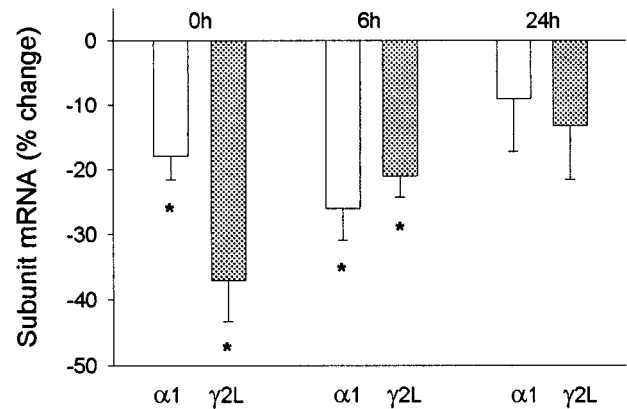
produced in peripheral organs may contribute to the amount of AP in the brain and to the physiological modulation of GABAergic synapses in various brain regions. Consistent with this conclusion, the marked decrease in the plasma concentration of progesterone at the end of pregnancy, as well as after adrenalectomy and orchiectomy, in rats is paralleled by a marked reduction in the brain content of AP (Paul and Purdy, 1992; Barbaccia et al., 1997; Concas et al., 1998). Moreover, adrenalectomy prevents the increases in the concentrations of progesterone and AP in both plasma and brain elicited by acute stress or by inhibitors of GABAergic transmission (Paul and Purdy, 1992; Barbaccia et al., 1997). Thus, the peripheral production of progesterone and its metabolism in the brain are likely critical determinants of



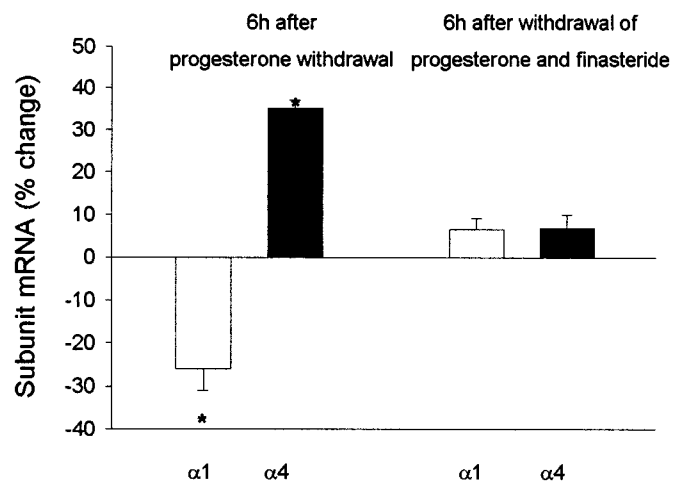
**Fig. 3.** Effect of finasteride on the progesterone-induced decrease in the abundance of GABA<sub>A</sub> receptor  $\gamma_2$  subunit transcripts. Cultured cerebellar granule cells were incubated for 5 days in the absence or presence of 1  $\mu$ M progesterone or 1  $\mu$ M finasteride as indicated, after which total RNA was extracted and subjected to RNase protection assay of the amounts of  $\gamma_{2L}$  (shaded bars) and  $\gamma_{2S}$  (open bars) transcripts. Data are expressed as the percentage of change relative to the values for control (vehicle-treated) cells and are means  $\pm$  S.E. ( $n = 9$ ) of values from three independent experiments. \* $P < .01$  versus control cells.



**Fig. 4.** Effect of progesterone withdrawal on the abundance of the GABA<sub>A</sub> receptor  $\alpha_4$  subunit mRNA. Cultured cerebellar granule cells were incubated for 5 days with 1  $\mu$ M progesterone and then for the indicated times in the absence of this steroid, after which total RNA was extracted and the amount of  $\alpha_4$  subunit mRNA was measured by RNase protection assay. Data are expressed as a percentage of values for control (vehicle-treated) cells and are means  $\pm$  S.E. ( $n = 4$  to 21) of values from seven independent experiments. \* $P < .01$  versus control cells.



**Fig. 5.** Effects of progesterone withdrawal on the abundance of GABA<sub>A</sub> receptor  $\alpha_1$  and  $\gamma_{2L}$  subunit mRNAs. Cultured cerebellar granule cells were incubated for 5 days in the presence of 1  $\mu$ M progesterone and then for 0, 6, or 24 h, as indicated, in the absence of this steroid, after which total RNA was extracted and the amounts of  $\alpha_1$  and  $\gamma_{2L}$  mRNAs were measured by RNase protection assay. Data are expressed as the percentage of change relative to values for control (vehicle-treated) cells and are means  $\pm$  S.E. ( $n = 7$  to 21 for  $\alpha_1$  mRNA,  $n = 3$  to 11 for  $\gamma_{2L}$  mRNA) of values from six independent experiments. \* $P < .01$  versus control values.



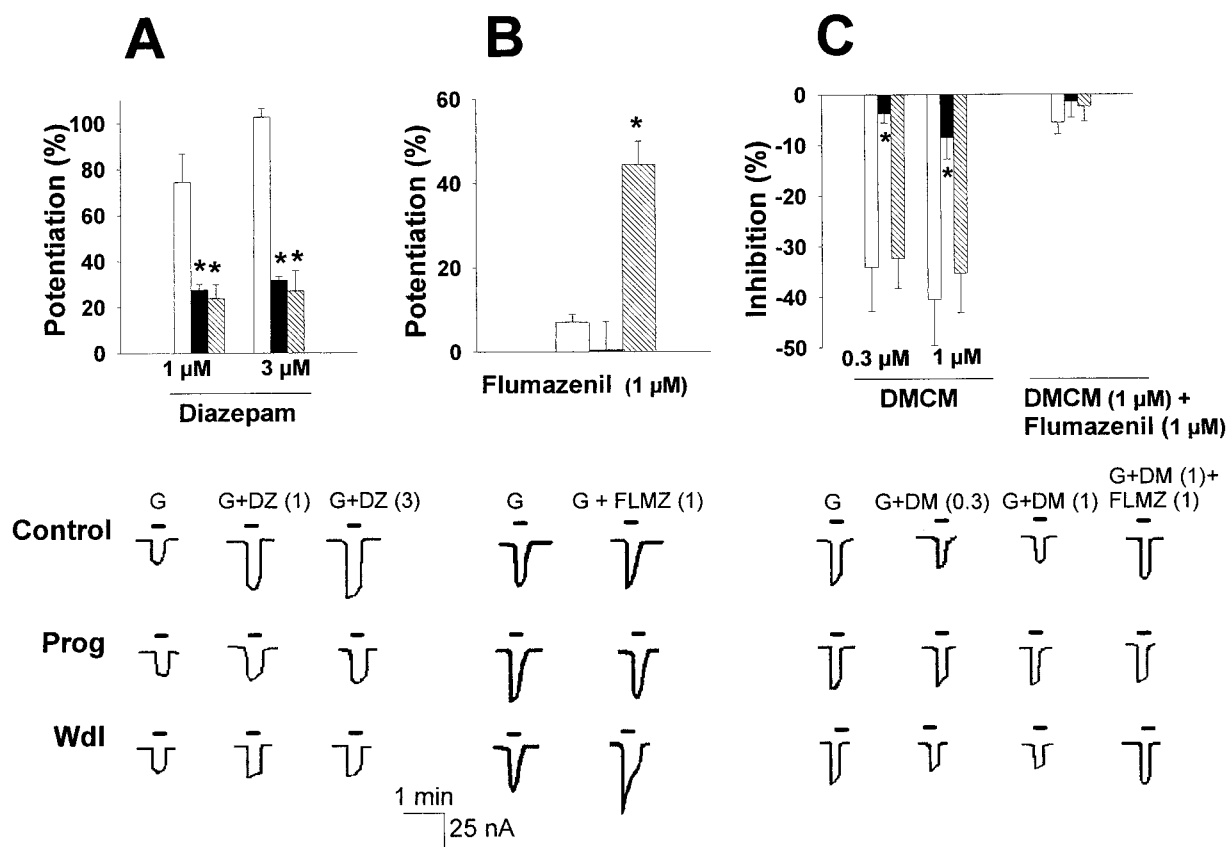
**Fig. 6.** Effect of finasteride on the progesterone withdrawal-induced initial increase in the abundance of the GABA<sub>A</sub> receptor  $\alpha_4$  subunit mRNA. Cultured cerebellar granule cells were incubated for 5 days with 1  $\mu$ M progesterone in the absence or presence of 1  $\mu$ M finasteride. After incubation of cells for an additional 6 h in the absence of drugs, total RNA was extracted and the amounts of  $\alpha_1$  and  $\alpha_4$  subunit mRNAs were measured by RNase protection assay. Data are expressed as the percentage of change relative to the values for control (vehicle-treated) cells and are means  $\pm$  S.E. ( $n = 7$  to 13) of values from four independent experiments. \* $P < .01$  versus control cells.

the central concentration of AP. Therefore, physiologically and pharmacologically induced fluctuations in progesterone production by the gonads or adrenal glands might affect the expression of specific GABA<sub>A</sub> receptor subunit genes and GABA<sub>A</sub> receptor activity in specific brain areas.

The discontinuation of long-term exposure of cultured granule cells to progesterone, with the consequent sudden decrease in the production of AP by these cells, resulted in a selective increase in the abundance of the GABA<sub>A</sub> receptor  $\alpha_4$  subunit mRNA. In contrast, the decreases in the amounts of  $\alpha_1$  and  $\gamma_{2L}$  subunit mRNAs elicited by persistent exposure to progesterone remained apparent 6 h after progesterone withdrawal. A similar effect of progesterone withdrawal was previously demonstrated in vivo with a pseudopregnancy model (Smith et al., 1998a,b). The presence of the  $\alpha_4$  subunit in recombinant GABA<sub>A</sub> receptors is associated with a reduced sensitivity to classical benzodiazepine agonists and zolpidem as well as with distinct patterns of regulation by flumazenil, DMCM, and other positive and negative modulators (Barnard et al., 1998). Electrophysiological recording of the pharmacological responses of GABA<sub>A</sub> receptors revealed that receptors derived from cells subjected to progesterone withdrawal were markedly less sensitive to the potentiating effect of diazepam than were those derived from control cells,

and they were positively modulated by flumazenil, the classical benzodiazepine receptor antagonist. These characteristics are compatible with those previously determined for  $\alpha_4$  subunit-containing GABA<sub>A</sub> receptors (Whittemore et al., 1996; Barnard et al., 1998). Moreover, withdrawal from chronic progesterone treatment restored the sensitivity of GABA<sub>A</sub> receptors to the inverse agonist DMCM, sensitivity that was markedly reduced during progesterone exposure. Thus, given that recombinant  $\alpha_4$  subunit-containing receptors, like  $\alpha_1$  subunit-containing receptors, are negatively modulated by DMCM (Whittemore et al., 1996), our data suggest that the increased sensitivity of GABA<sub>A</sub> receptors to DMCM after progesterone withdrawal is attributable to the increase in  $\alpha_4$  subunit expression. It is possible that an increased sensitivity to endogenous inverse agonists may contribute to the etiology of progesterone withdrawal syndrome.

These observations, together with the evidence that the abundance of the  $\alpha_4$  subunit mRNA was unchanged during chronic exposure of granule cells to progesterone, suggest that the  $\alpha_4$  subunit contributes to changes in the sensitivity of GABA<sub>A</sub> receptors to both drugs and endogenous modulators, as well as to consequent changes in receptor activity and behavior, that are specific to physiological and pathological conditions associated with rapid and marked decreases in the



**Fig. 7.** Effects of long-term progesterone treatment and progesterone withdrawal on the modulation of GABA<sub>A</sub> receptor function by various benzodiazepine receptor ligands. *Xenopus* oocytes were injected with crude membranes prepared from control (vehicle-treated) cerebellar granule cells (light gray columns), from granule cells treated with 1  $\mu$ M progesterone for 5 days (black columns), or from granule cells incubated with 1  $\mu$ M progesterone for 5 days and then in the absence of the steroid for 6 h (dark gray columns). The effects of the indicated concentrations of diazepam (A), flumazenil (B), or DMCM in the absence or presence of flumazenil (C) on the amplitude of the Cl<sup>-</sup> current induced by GABA (at a concentration yielding 10% of the maximal response) were examined. Top, data are expressed as percentage of potentiation or percentage of inhibition of the response to GABA and are means  $\pm$  S.E. of values obtained from three to seven different oocytes. \**P* < .05 versus receptors from control cells. Bottom, representative electrophysiological tracings obtained from individual oocytes for each experimental group. Prog, chronic progesterone treatment; Wdl, progesterone withdrawal; G, GABA; DZ, diazepam; FLMZ, flumazenil; DM, DMCM; numbers in parentheses, concentration in micromolar.



plasma and brain concentrations of progesterone. Thus, the increase in the abundance of the  $\alpha_4$  subunit mRNA during withdrawal from progesterone in the rat pseudopregnancy model is associated with changes in the kinetics of hippocampal GABA<sub>A</sub> receptor-mediated currents, with experimental anxiety, and with increased seizure susceptibility (Smith et al., 1998b).

Systemic administration of progesterone induces anxiolytic and anticonvulsant effects. These effects are temporally and functionally correlated with the brain content of AP and the state of activation of GABA<sub>A</sub> receptors (Bitran et al., 1995). Our results suggest that the production of AP as a result of neuronal metabolism of progesterone generated in the periphery might be an important determinant of GABA<sub>A</sub> receptor function and plasticity as well as of associated behavior. The observation that the effects of progesterone withdrawal were prevented by administration of finasteride together with the progesterone further suggests that the withdrawal-induced increase in the abundance of the  $\alpha_4$  subunit mRNA is triggered by the associated decrease in AP synthesis. This conclusion is consistent with our previous demonstration that, by reversing the pregnancy-induced increase in the brain content of AP, finasteride antagonized the decrease in GABA<sub>A</sub> receptor function and changes in the abundance of the  $\gamma_{2L}$  subunit mRNA normally observed during pregnancy (Concas et al., 1998).

Evidence thus suggests that changes in the physiological rhythm of progesterone secretion that accompany conditions such as pregnancy, the estrous cycle, menopause, aging, and chronic stress, together with the neuronal metabolism of this steroid to AP and consequent modulation of GABA<sub>A</sub> receptor gene expression and receptor function, may contribute to the development of mental diseases often associated with such conditions. For example, the sudden and rapid decrease in peripheral and central concentrations of progesterone during the menstrual cycle may contribute to mental symptoms associated with premenstrual syndrome, and the marked decrease in the secretion of steroid hormones that occurs during menopause may be important in the development of such symptoms in postmenopausal women (Schmidt et al., 1994; Wang et al., 1996; Rapkin et al., 1997; Bickova et al., 1998; Genazzani et al., 1998). Moreover, given that AP exhibits pharmacological and biochemical profiles (Majewska et al., 1986) similar to those of benzodiazepines and barbiturates, an increase in the abundance of the  $\alpha_4$  subunit might be a common mechanism underlying the development of tolerance, dependence, and withdrawal syndrome associated with long-term therapy and discontinuation of treatment with anxiolytic and hypnotic drugs.

In conclusion, our demonstration that granule cells in culture are able to metabolize progesterone to AP suggests that the peripheral secretion of progesterone and its metabolism by neurons and glia in the brain may play a physiological role in the modulation of those aspects of brain function (emotion, mood, cognition) that are regulated by GABAergic synapses (Biggio et al., 1995; Sieghart, 1995).

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