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Physiological modulation of $GABA_A$ receptor plasticity by progesterone metabolites

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Abstract

The possible functional relation between changes in brain and plasma concentrations of neurosteroids and the plasticity of γ-aminobutyric acid type A ($GABA_A$) receptors in the brain during pregnancy and after delivery was investigated in rats. The concentrations in the cerebral cortex and plasma of pregnenolone as well as of progesterone and its neuroactive derivatives allopregnanolone (3α-hydroxy-5α-pregnan-20-one) and allotetrahydrodeoxycorticosterone (5α-hydroxy-3α,21-diol-20-one) increased during pregnancy, peaking around day 19, before returning to control (estrus) values immediately before delivery (day 21). In the postpartum period, steroid concentrations in plasma and brain did not differ from control values. The densities of [^{3}H]GABA, [^{3}H]flunitrazepam, and *t*-[^{35}S]butylbiciclophosphorotionate (TBPS) binding sites in the cerebral cortex also increased during pregnancy, again peaking on day 19 and returning to control values on day 21; receptor density was decreased further 2 days after delivery and again returned to control values within 7 days. These changes were accompanied by a decrease in the apparent affinity of the binding sites for the corresponding ligand on day 19 of pregnancy. The amount of the γ2L subunit mRNA decreased progressively during pregnancy, in the cerebral cortex and hippocampus, returned to control value around the time of delivery and did not change in the postpartum period. On the contrary, the amount of α4 subunit mRNA was not modified during pregnancy both in the cerebral cortex and hippocampus whereas significantly increased 7 days after delivery only in the hippocampus. No significant changes were apparent for α1, α2, α3, β1, β2, β3 and γ2S subunit mRNAs. Administration of finasteride, a specific 5α-reductase inhibitor, to pregnant rats from days 12 to 18 markedly reduced the increases in the plasma and brain concentrations of allopregnanolone and allotetrahydrodeoxycorticosterone as well as prevented both the increase in the densities of [^{3}H]flunitrazepam and [^{35}S]TBPS binding sites and the decrease of γ2L mRNA normally observed during pregnancy. The results demonstrate that the changes in the plasticity of $GABA_A$ receptors that occur in rat brain during pregnancy and after delivery are related to the physiological changes in plasma and brain concentrations of neurosteroids.

Keywords: Allopregnanolone; Allotetrahydrodeoxycorticosterone; $GABA_A$ receptor; Pregnancy; Finasteride; Brain, rat

1. Introduction

Systemic administration of progesterone or its metabolites 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) and 5α-hydroxy-3α,21-diol-20-one (allotetrahydrodeoxycorticosterone) share whit benzodiazepines and barbiturates the capability to induce anxiolytic (Crawley et al., 1986; Bitran et al., 1991), hypnotic (Mendelson et al., 1987) or anticonvulsant (Belelli et al., 1990; Kokate et al., 1994; Concas et al., 1996) effects and to enhance the function of $GABA_A$ receptors (Majewska, 1992; Lambert et al., 1995). Accordingly, pharmacological treatments, such as the oral administration of progesterone to healthy female volunteers (Freeman et al., 1993; McAuley et al., 1995) and the intracerebroventricular or parental administration of progesterone, allopregnanolone, or allotetrahydrodeoxycorticosterone to rats (Crawley et al., 1986; Belelli et al., 1990; Bitran et al., 1991; Kokate et al., 1994; Concas et al., 1996) induced parallel alterations in the

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behavior related to $GABA_A$ receptors function. These clinical and experimental data have suggested that fluctuations in plasma and brain concentrations of neuroactive steroids induced by physiological, pharmacological or pathological conditions might affect $GABA_A$ receptor function and this may result in alterations in the emotional state, sleep pattern and seizure threshold. Thus, the marked fluctuations in the plasma and brain concentrations of these compounds associated with physiological conditions such as acute and chronic stress, pregnancy, postpartum, estrous cycle, menopause, etc. (Purdy et al., 1991; Barbaccia et al., 1994, 1996, 1997; Finn and Gee, 1994; Halbreich et al., 1996; Rapkin et al., 1997; Concas et al., 1998; Biggio et al., 1999) may play an important role for the development of some disorders (anxiety, depression, premenstrual syndrome, etc.) associated to such conditions.

To clarify the role of these neuroactive steroids in the modulation of the $GABA_A$ receptor activity, we have now evaluated the possible functional relation between the plasticity of $GABA_A$ receptors and the variations in plasma and brain concentrations of progesterone, allopregnanolone and allotetrahydrodeoxycorticosterone associated with a physiological condition in which both $GABA_A$ receptors and neurosteroids undergo marked and dynamic changes. Thus, we investigated in rats whether the increase in the concentrations of neuroactive steroids that occurs in plasma and brain during pregnancy may exert a tonic modulatory action on the density and gene expression of $GABA_A$ receptors in the cerebral cortex and hippocampus. Moreover, we also evaluated whether the sudden decrease in the concentrations of these compounds immediately before parturition and their low abundance during lactation may represent a withdrawal-like phenomenon. Finally, to prove the physiological role of brain levels of allopregnanolone and allotetrahydrodeoxycorticosterone in the modulation of $GABA_A$ receptor function and plasticity during pregnancy, we used finasteride, a selective blocker of 5α-reductase (Rittmaster, 1994), to abolish the marked increase of brain allopregnanolone and allotetrahydrodeoxycorticosterone during the last week of pregnancy.

2. Materials and methods

2.1. Animals

Adult female Sprague–Dawley rats (Charles River, Como, Italy) with body masses of 200 to 250 g were studied. After arrival at the animal facility, rats were acclimatized to the new housing conditions for at the least 1 week. The animals were housed six per cage under an artificial 12-h light, 12-h dark cycle (light on from 0800 to 2000 h) at a constant temperature of 22 ± 2°C and a relative humidity of 65%. They had free access to water and standard laboratory food throughout the entire experimental period.

Stage of estrous cycle (diestrus, proestrus, or estrus) was determined from daily vaginal smears taken between 0900 and 1000 h for 2 to 4 weeks. Only rats exhibiting at least three regular cycles were studied further. For the induction of pregnancy, females were caged with proven males on the evening of proestrus. Mating was verified by observation of spermatozoa in the vaginal smear taken next morning, which was designated day 0 of pregnancy. Pregnancy was confirmed by additional vaginal smears or the presence of embryos.

In one series of experiments, pregnant rats were injected subcutaneously at the nape of the neck with finasteride (25 mg/kg body mass) or vehicle once a day (1300 h) from days 12 to 18 of gestation. Dams were killed on day 19. Finasteride was dissolved in a mixture of ethanol (20%, v/v) and corn oil (80%) and injected in a volume of 3 ml/kg. Control dams received the same amount of vehicle.

2.2. Steroid extraction and assay

Animals were killed either by guillotine (for plasma steroid measurements) or by focused microwave irradiation (70 W/cm for 4 s) to the head (for brain steroid measurements). This latter procedure results in a virtually instantaneous inactivation of brain enzymes, thus minimizing postmortem steroid metabolism. Brains were rapidly (< 1 min) removed, and the cerebral cortices were dissected and frozen to -20°C until steroid extraction. Blood was collected from the trunk into heparinized tubes and centrifuged at $900 \times g$ for 20 min, after which the plasma was frozen until assayed for steroids.

Steroids were extracted and purified as previously described (Barbaccia et al., 1996). Briefly, steroids present in cerebral cortical homogenates (400 mg of tissue in 4 ml of phosphate-buffered saline) were extracted three times with an equal volume of ethyl acetate. The organic phases were dried under vacuum, the residue was dissolved in 5 ml of *n*-hexane and applied to Seppak-silica cartridges (Waters), and components were eluted with *n*-hexane/2-propanol (7:3, v/v). Steroids were further purified by high-performance liquid chromatography on a 5-μm Lichrosorb-diol column (250 by 4 mm) (Merck) with a gradient of 2-propanol in *n*-hexane. Because we previously observed that cholesterol, which coelutes from the Lichrosorb-diol column with progesterone, reduces the sensitivity of the radioimmunoassay (RIA) for progesterone, this latter steroid was separated from cholesterol by washing the corresponding dried column fractions twice with 200 μl of dimethylsulphoxide and 400 μl of water. Progesterone was extracted from the aqueous phase twice with 1.5 ml of *n*-hexane. The removal of cholesterol increased the sensitivity of the progesterone RIA 5- to 10-fold relative to that previously described (Barbaccia et al., 1996). The recovery

of each steroid through the extraction–purification procedures (70 to 80%) was monitored by adding trace amounts (4000 to 6000 cpm) of ^{3}H-labeled standards to the brain tissue homogenate. Allopregnanolone and allotetrahydrodeoxycorticosterone were then quantified by RIA as previously described (Purdy et al., 1990), and other steroids were measured by RIA with specific antibodies (ICN, Costa Mesa, CA).

Protein concentration was measured as described (Lowry et al., 1951) with bovine serum albumin as standard. Plasma concentrations were measured in 1 ml of plasma after extraction three times with 1.5 ml of ethyl acetate.

2.3. [^{3}H]GABA binding assay

Cerebral cortices were homogenized with a Polytron PT 10 (setting 5 for 30 s) in 10 vols. of ice-cold water. The homogenate was centrifuged at 48,000 × *g* for 10 min at 0°C, and the resulting pellet was washed once by resuspension and recentrifugation in 10 vols. of 20 mM potassium phosphate buffer (pH 7.4) containing 50 mM KCl. The membranes were stored at −20°C until use 1 to 15 days later. On the day of the assay the membranes were thawed, centrifuged, and washed three additional times by resuspension and recentrifugation in ice-cold buffer. The membranes were then resuspended in 50 vols. of the same buffer, and 300 μl of the resulting suspension (0.2 to 0.3 mg of protein) were added to plastic minivials. Incubation for 10 min at 4°C in a total volume of 0.5 ml was initiated by the addition of [^{3}H]GABA and was stopped by centrifugation at 48,000 × *g* for 10 min. The resulting supernatant was discarded and the pellet was washed gently twice with 4 ml of ice-cold distilled water and was then dissolved in 3 ml of scintillation fluid (Atomlight; New England Nuclear, Boston, MA). Nonspecific binding was defined as binding in the presence of 1 mM unlabeled GABA. Saturation analysis of [^{3}H]GABA binding was performed with 12 different concentrations (10 to 1800 nM) of ligand; the concentration of radioligand was maintained constant at 10 nM and diluted with unlabeled GABA. Scatchard analysis of the binding data was performed with an iterative computer program (LIGAND), which accepts the binding data (bound and bound/free) as input and determines whether the results from individual Scatchard plots are best fitted by a one- or a two-site model. This analysis provides estimates for the dissociation constant (K_D) and maximal number of binding sites (B_{max}).

2.4. [^{3}H]Flunitrazepam binding assay

Cerebral cortices were homogenized with a Polytron PT 10 (setting 5 for 30 s) in 10 vols. of ice-cold water and the homogenate was centrifuged for 10 min at 48,000 × *g* and 4°C. The resulting pellet was washed three times by resuspension and recentrifugation (10 min at 48,000 × *g* and 4°C) in 50 vols. of 50 mM Tris–HCl (pH 7.4), and was finally reconstituted in 50 vols. of the same buffer. The membranes were stored at −20°C until use 1 to 15 days later. On the day of the assay, the membranes were thawed, centrifuged, and resuspended in 50 vols. of buffer. [^{3}H]Flunitrazepam binding was measured in a final volume of 500 μl, consisting of 200 μl of membranes (0.15 to 0.20 mg of protein), 50 μl of [^{3}H]flunitrazepam and buffer to volume. Incubations (0°C) were initiated by addition of membranes and terminated after 60 min by rapid filtration through glass-fiber strips (Whatman GF/B). The filters were rinsed with two 4-ml portions of ice-cold Tris–HCl buffer in a Cell Harvester filtration manifold (Model M-24; Brandel Instruments, Gaithersburgh, MD), and filter-bound radioactivity was quantitated by scintillation spectroscopy. Nonspecific binding was defined as binding in the presence of 5 μM diazepam. Saturation analysis of [^{3}H]flunitrazepam binding was performed with eight different concentrations of radioligand (0.125 to 16 nM).

2.5. [^{35}S]TBPS binding assay

Cerebral cortices were homogenized with a Polytron PT 10 (setting 5 for 30 s) in 75 vols. of ice-cold 50 mM Tris–citrate buffer (pH 7.4 at 25°C) containing 100 mM NaCl. The homogenate was centrifuged at 20,000 × *g* for 20 min, and the resulting pellet was resuspended in 75 vols. of 50 mM Tris–citrate buffer without salt and used for the assay. *t*-[^{35}S]Butylbicyclophosphorothionate (TBPS) binding was determined in a final volume of 500 μl, consisting of 200 μl of membranes (0.15 to 0.20 mg of protein), 50 μl of [^{35}S]TBPS, 50 μl of 2 M NaCl, and 200 μl of 50 mM Tris–citrate buffer. Incubations (25°C) were initiated by addition of membranes and terminated after 90 min by rapid filtration through glass-fiber strips (Whatman GF/B). The filters were rinsed with two 4-ml portions of ice-cold Tris–citrate buffer in a Cell Harvester filtration manifold, and filter-bound radioactivity was quantitated by scintillation spectroscopy. Nonspecific binding was defined as binding in the presence of 100 μM picrotoxin. Saturation analysis of [^{35}S]TBPS binding was performed with seven different concentrations of ligand (2.5 to 500 nM); the concentration of radioligand was maintained constant at 2.5 nM [^{35}S]TBPS and diluted with unlabeled TBPS.

2.6. Reverse transcription–polymerase chain reaction

Total RNA was extracted from rat brain (Follesa and Ticku, 1996; Yu et al., 1996) and subjected to reverse transcription with SuperScript reverse transcriptase (Life Technologies, Gaithersburg, MD, USA) in the presence of oligo(dT). The resulting cDNA (1–10 ng) was then ampli-

Table 1
Effect of pregnancy on dissociation costant (K_D) of [^{3}H]GABA, [^{3}H]FLU, and [^{35}S]TBPS binding to membranes preparation from rat cerebral cortex

	[^{3}H]GABA binding	[^{3}H]FLU binding	[^{35}S]TBPS binding
	K_D (nM)	K_D (nM)	K_D (nM)
Estrus	796 ± 51	2.56 ± 0.19	106 ± 4.3
Day 10 of pregnancy	803 ± 56	2.78 ± 0.22	116 ± 8.6
Day 15 of pregnancy	915 ± 69	3.38 ± 0.26[a]	144 ± 13.3[a]
Day 19 of pregnancy	987 ± 47[a]	3.41 ± 0.28[a]	145 ± 10[a]
Day 21 of pregnancy	818 ± 45	2.93 ± 0.16	100 ± 11.5
2 days after delivery	689 ± 54	2.07 ± 0.13	91.5 ± 5.15

Binding assays were performed as described in the text. Increasing concentration of [^{3}H]GABA (10 to 1800 nM), [^{3}H]FLU (0.125 to 16 nM) and [^{35}S]TBPS (2.5 to 500 nM) were used and each determination was done in triplicate. The K_D values represent means ± S.E.M. from four to six experiments.
[a] $P < 0.05$ vs. estrus females.

fied by the polymerase chain reaction (Follesa and Ticku, 1996; Yu et al., 1996) with *Taq* DNA polymerase (2.5 U) (Perkin-Elmer/Cetus, Norwalk, CT, USA) in 100 μl of standard buffer [100 mM Tris–HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin] containing 1 mM sense and antisense primers specific for various subunits of the $GABA_A$ receptor and 200 mM of each deoxynucleoside triphosphate. The various primer pairs (Table 1) were designed to include cDNA sequences with the lowest degree of homology among the different receptor subunits (Follesa and Ticku, 1996; Yu et al., 1996). Reaction was performed in a thermal cycler (Ericomp, San Diego, CA, USA) 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 and Ticku, 1996). 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, Gaithersburg, MD, USA). *Escherichia coli* NM522 was transformed with the resulting plasmids, which were subsequently purified from the bacteria and the cDNA inserts sequenced with a Sequenase DNA sequencing kit (USB, Cleveland, OH, USA). The nucleotide sequences were 100% identical to the respective previously published sequences (see Table 1 for references).

2.7. Probe preparation

Plasmids containing the cDNA fragments corresponding to the various $GABA_A$ receptor subunits were linearized with restriction enzymes (Table 2) and used as a template, together with the appropriate RNA polymerase (SP6 or T7), to generate [α-^{32}P]CTP-labeled cRNA probes for RNase protection assays.

2.8. RNase protection assay

RNase protection assay is a sensitive technique for semiquantitative detection of RNA (Zinn et al., 1983; Lee and Costlow, 1987). RNase protection assays were performed as previously described (Follesa and Ticku, 1996). Total RNA was extracted (Follesa and Ticku, 1996) from rat total cerebral cortex or hippocampus and quantified by measurement of absorbance at 260 nm. Briefly, 25 μg of total RNA were dissolved in 20 μl of hybridization solution containing 150,000 cpm of ^{32}P-labeled cRNA probe encoding the specific $GABA_A$ receptor subunits (specific activity, 6×10^7 to 7×10^7 cpm/μg) and 15,000 cpm of ^{32}P-labeled internal standard cyclophilin cRNA (specific activity, 1×10^6 cpm/μg). Cyclophilin is a ubiquitous protein expressed also in the brain and, its gene is most likely to be regulated in an «on or off» fashion (Milner and Sutcliffe, 1983; Danielson et al., 1988) therefore it can be used as internal standard in our measurement. The samples were then incubated at 50°C overnight and subjected to digestion with RNase, after which RNA–RNA hybrids were separated by electrophoresis on a sequencing gel containing 5% polyacrylamide and urea and visualized by autoradiography. The content of $GABA_A$ receptor subunit mRNAs and cyclophilin mRNA were evaluated, as previously published (Follesa and Ticku, 1996), by measuring the optical density of the autoradiography bands for each lane with a Bio-Rad GS-700 Imaging Densitometer,

Table 2
Effect of finasteride on [^{3}H]FLU and [^{35}S]TBPS binding to cerebral cortex of pregnant rats

Treatment	[^{3}H]FLU binding		[^{35}S]TBPS binding	
	B_{max} (pmol/mg protein)	K_D (nM)	B_{max} (pmol/mg protein)	K_D (nM)
Estrus	1.24 ± 0.09	2.7 ± 0.20	2.15 ± 0.15	93 ± 4.2
Pregnant	1.65 ± 0.11[a]	3.5 ± 0.22[a]	3.08 ± 0.24[a]	130 ± 6.6[a]
Pregnant + finasteride	1.31 ± 0.11	3.0 ± 0.19	2.07 ± 0.27	104 ± 7.1

Finasteride (25 mg/kg, s.c.) or solvent were injected daily starting on day 12 through day 18 of pregnancy and rats were killed on day 19. Saturation experiments were performed using eight concentrations (0.125 to 16 nM) of [^{3}H]FLU and seven concentrations (2.5 to 500 nM) of [^{35}S]TBPS. Data are the means ± S.E.M. of four experiments, each performed in triplicate.
[a] $P < 0.05$ vs. estrus value.

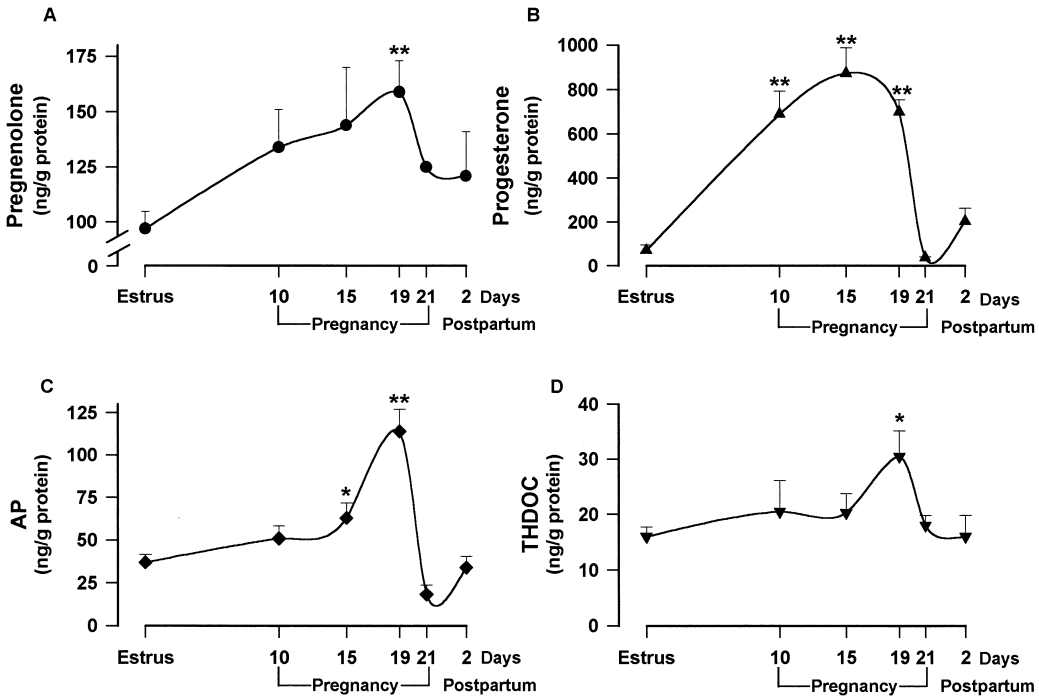

Fig. 1. Changes in cerebrocortical neurosteroid concentrations during pregnancy and after delivery. Values are expressed as nanograms of steroid per gram of cortical protein and are means ± S.E.M. of values obtained from 8 to 10 rats. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. estrus.

(Bio-Rad Laboratories, Hercules, CA, USA) this instrument is calibrated so that saturated values are detected, therefore all our measurement are in the linear range. The data were normalized by dividing the optical density value of each protected fragment by the optical density value of the respective protected fragment band of the cyclophilin. Therefore, the amount of mRNA was expressed in arbitrary units (ratio between optical density).

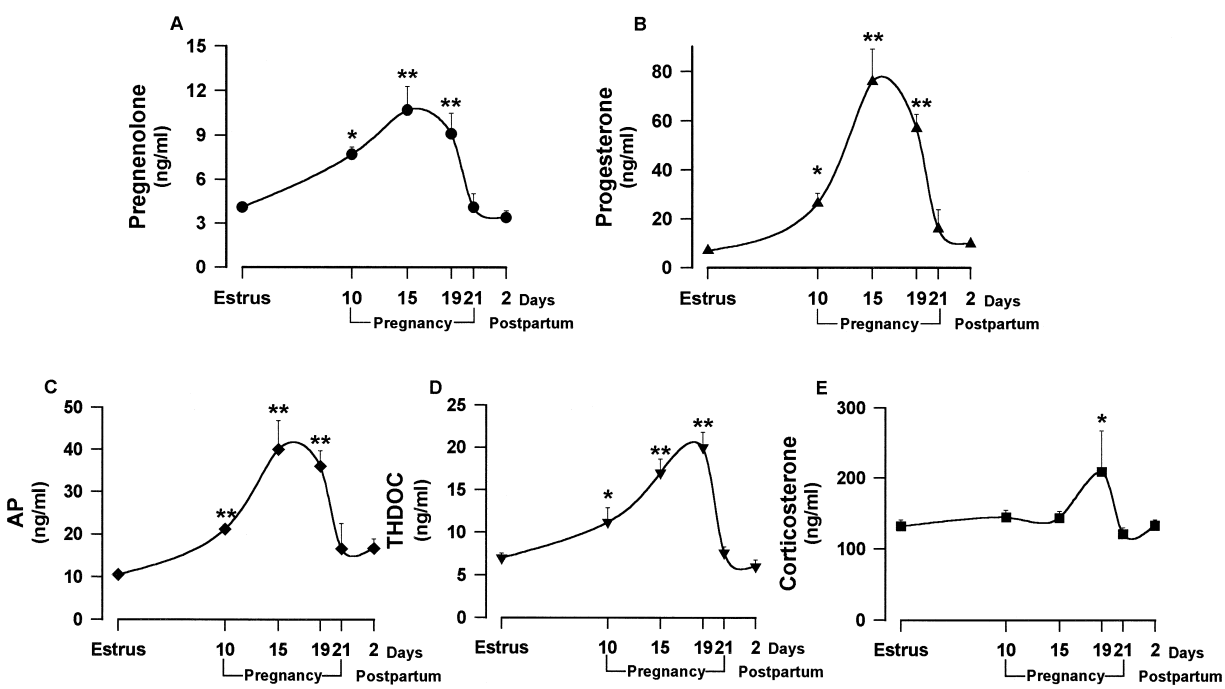

Fig. 2. Changes in plasma neurosteroid concentrations during pregnancy and after delivery. Values are expressed as nanograms of steroid per milliliter of plasma and are the means ± S.E.M. of values obtained from 8 to 10 rats. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. estrus.

2.9. Statistical analysis

Data are presented as means ± S.E.M. The statistical significance of differences was assessed by analysis of variance followed by Scheffe's test. A P-value of < 0.05 was considered statistically significant.

3. Results

3.1. Steroid concentrations in plasma and cerebral cortex

The changes in the concentrations of pregnane steroids in the rat cerebral cortex during pregnancy and after delivery, compared with the corresponding concentrations on the day of estrus (control values), are shown in Fig. 1. The concentration of progesterone was increased markedly (9-fold) on day 10 of pregnancy, reached a peak (12-fold) on day 15, was still higher (10-fold) than the estrus value on day 19, and returned to control values immediately before delivery (day 21). The concentrations of pregnenolone and allopregnanolone were not significantly changed on day 10 of pregnancy, showed a maximal increase (+63 and +208%, respectively) on day 19, and returned to control values on day 21. In contrast, the concentration of allotetrahydrodeoxycorticosterone in the cerebral cortex remained unchanged during the first 15 days of pregnancy, was significantly increased (+90%) on day 19, and had returned to control values on day 21. The cortical concentrations of all steroids during the postpartum period did not differ from control values.

The time courses of changes in steroid concentrations in plasma during pregnancy and after parturition were more similar than those in brain steroid concentrations (Fig. 2). Plasma concentrations of pregnenolone, progesterone, allopregnanolone and allotetrahydrodeoxycorticosterone were significantly increased (+87, +274, +114, and +77%, respectively) 10 days after copulation, were further increased (~2.5-, 10-, 3.6-, and 2.5-fold the estrus value, respectively) on days 15 to 19 of pregnancy, and had returned to control values on day 21, thereafter remaining unchanged for up to 7 days after parturition (data not shown). The plasma concentration of corticosterone, the major adrenal corticosteroid in the rat, was significantly increased (+58%) only on day 19 of pregnancy.

3.2. Effects of pregnancy on [^{3}H]GABA, [^{3}H]flunitrazepam, and [^{35}S]TBPS binding

Changes in the binding of [^{3}H]GABA, [^{3}H]flunitrazepam, and [^{35}S]TBPS to cerebral cortical membranes during pregnancy and after parturition are shown in Fig. 3 and Table 1. Saturation analysis of [^{3}H]GABA binding with the LIGAND program revealed two populations of recognition sites in the brain of nonpregnant female rats: a high-affinity site (B_{max1}, 0.52 ± 0.06 pmol/mg protein; K_{D1}, 19 ± 2 nM) and a low-affinity site (B_{max2}, 5.04 ± 0.70 pmol/mg protein; K_{D2}, 796 ± 51 nM). Whereas the equilibrium binding parameters of the high-affinity site did not change significantly during pregnancy and after parturition (data not shown), the density of low-affinity $GABA_A$ receptors was increased significantly (+19%), with respect to the value for nonpregnant females, on day 15 of pregnancy, reached a maximum (+26%) on day 19, and had returned to control values immediately before parturition (day 21). The density of these binding sites was decreased (−27%) below control values 2 days after parturition, before returning to control levels by 7 days

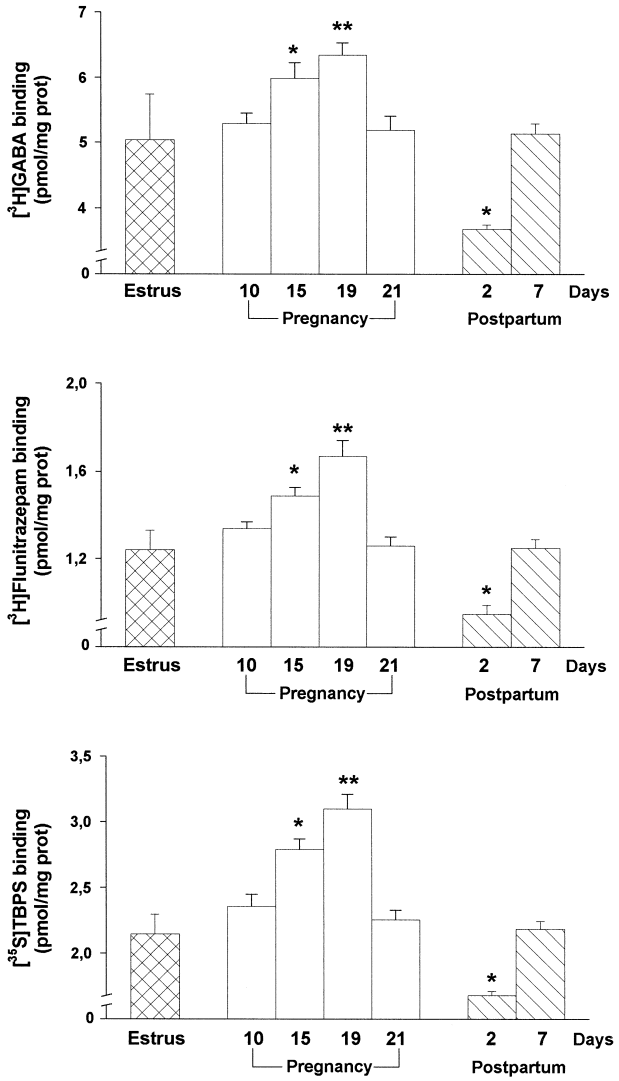

Fig. 3. Changes in the density of [^{3}H]GABA (A), [^{3}H]FLU (B) and [^{35}S]TBPS (C) binding sites during pregnancy and after delivery. Saturation experiments were performed using 12 concentrations (10 to 1800 nM) of [^{3}H]GABA, eight concentrations (0.125 to 16 nM) of [^{3}H]FLU and seven concentrations (2.5 to 500 nM) of [^{35}S]TBPS. Data are expressed as the percentage of changes with respect to estrus value and are the means ± S.E.M. of four to six experiments, each performed in triplicate. The B_{max} values (pmol/mg protein) for estrus rats were 5.04 ± 0.7 ([^{3}H]GABA), 1.52 ± 0.08 ([^{3}H]FLU) and 2.13 ± 0.5 ([^{35}S]TBPS). $^*P < 0.05$; $^{**}P < 0.01$ vs. estrus value.

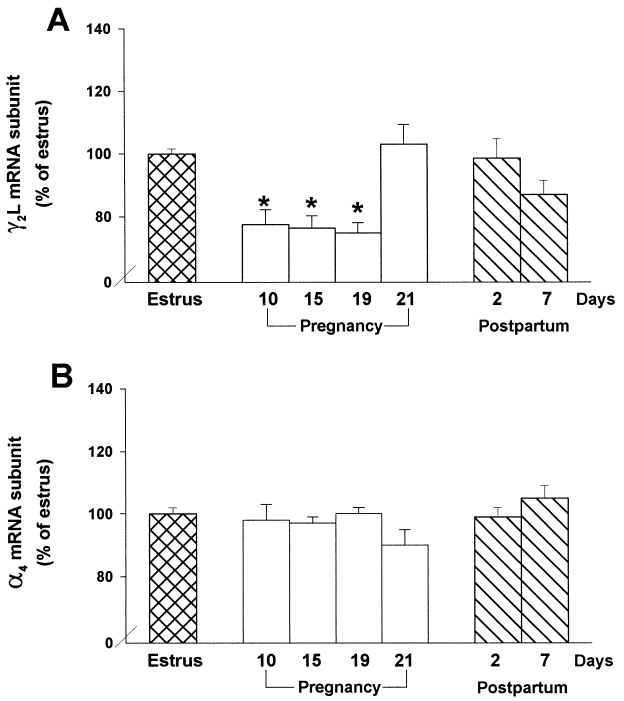

Fig. 4. Effect of pregnancy on the amounts of γ2L (A) and α4 (B) subunit mRNAs in the cerebral cortex as measured by RNase protection assay. Data are expressed as a percentage of the value for control rats in estrus and are means ± S.E.M. of 9 to 30 animals. $^{*}P < 0.05$ vs. estrus value.

after delivery (Fig. 3A). These changes in receptor density were accompanied by a significant increase (+24%) in the corresponding K_D on day 19 of pregnancy (Table 1).

The binding of the benzodiazepine [^{3}H]flunitrazepam to cerebrocortical membranes showed a similar pattern of changes to those of [^{3}H]GABA binding to the low-affinity sites. The increase in benzodiazepine receptor density peaked (+35%) on day 19 of pregnancy, returned to control values on day 21 of pregnancy, and was significantly decreased (−23%) 2 days after delivery (Fig. 3B). Moreover, the corresponding K_D value was increased by 32 and 33% on days 15 and 19 of pregnancy, respectively (Table 1).

The cortical density of binding sites for [^{35}S]TBPS, a selective ligand for the $GABA_A$ receptor-coupled Cl^- channel, was significantly increased (+30%) on day 15 of pregnancy, was maximal (+44%) on day 19, and had returned to control values on day 21. The density was decreased (−22%) 2 days postpartum and had returned again to control values by 7 days after delivery (Fig. 3C). The corresponding K_D value was increased by ~36% on days 15 to 19 of pregnancy (Table 1).

3.3. Effects of pregnancy on the expression of $GABA_A$ receptor subunit mRNAs

The amounts of γ2L subunit mRNA, measured by RNase protection assay, decreased progressively during pregnancy in the cerebral cortex and hippocampus. In the former brain region, the decrease was apparent (−23 ± 4%) on day 10 of pregnancy, peaked (−26 ± 3%) on day 19 and returned to control values on day 21 (Fig. 4A). In the hippocampus, the expression of this subunit mRNA remained unchanged on day 10 but was reduced on day 15 (−30 ± 3%) and on day 19 (−23 ± 3%) compared with the estrus value (Fig. 5A). The amounts of γ2L subunit mRNA during the postpartum period did not differ significantly from control (estrus) values in both cerebral cortex and hippocampus.

In the same animals, the abundance of mRNA encoding the α4 subunit of the $GABA_A$ receptor specifically increased (+30 ± 6%) in the hippocampus (Fig. 5B) only on the seventh day after delivery, while remaining unchanged in the cerebral cortex (Fig. 4B). No significant changes were apparent for α1, α2, α3, β1, β2, β3 and γ2S subunit mRNAs in both brain regions during pregnancy and after delivery.

3.4. Effects of chronic blockade of 5α-reductase on neurosteroids and $GABA_A$ receptors

To clarify further the role of allopregnanolone and allotetrahydrodeoxycorticosterone in the changes of $GABA_A$ receptor plasticity during pregnancy we investigated whether a reduction in the brain content of these neurosteroids elicited by the administration to pregnant rats of finasteride, a specific 5α-reductase inhibitor (Rittmaster, 1994), could antagonize the changes of

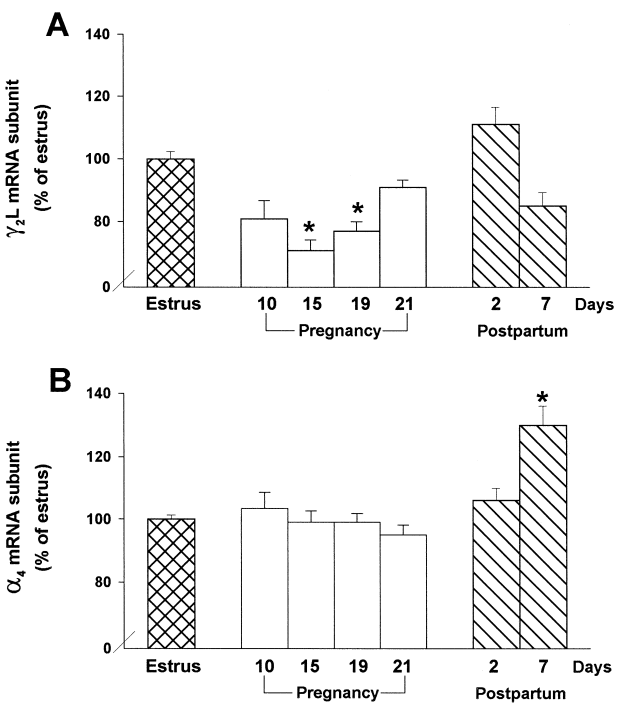

Fig. 5. Effect of pregnancy on the amounts of γ2L (A) and α4 (B) subunit mRNAs in the hippocampus as measured by RNase protection assay. Data are expressed as a percentage of the value for control rats in estrus and are means ± S.E.M. of 9 to 30 animals. $^{*}P < 0.05$ vs. estrus value.

[^{3}H]flunitrazepam and [^{35}S]TBPS binding and the gene expression of γ2L receptor subunit. Administration of this drug (25 mg/kg) from days 12 to 18 of pregnancy markedly reduced the pregnancy-induced increases in the concentrations of allopregnanolone and allotetrahydrodeoxycorticosterone in both the cerebral cortex and plasma (Fig. 6). Moreover, consistent with the previous (Concas et al., 1998) finding showing that finasteride prevented the decrease in the stimulatory effect of muscimol on $^{36}Cl^-$ uptake at day 19 of pregnancy, this 5α-reductase inhibitor abolished the increases in both the density (B_{max}) and K_D of [^{3}H]flunitrazepam and [^{35}S]TBPS binding sites normally apparent on day 19 of pregnancy (Table 2). Neither the addition of finasteride to the in vitro assays nor its acute administration (25 mg/kg, s.c.) in vivo affected [^{3}H]flunitrazepam or [^{35}S]TBPS binding (data not shown). Finally, finasteride also prevented the pregnancy-induced decrease in the expression of γ2L subunit mRNA both in the cerebral cortex and hippocampus (Table 3).

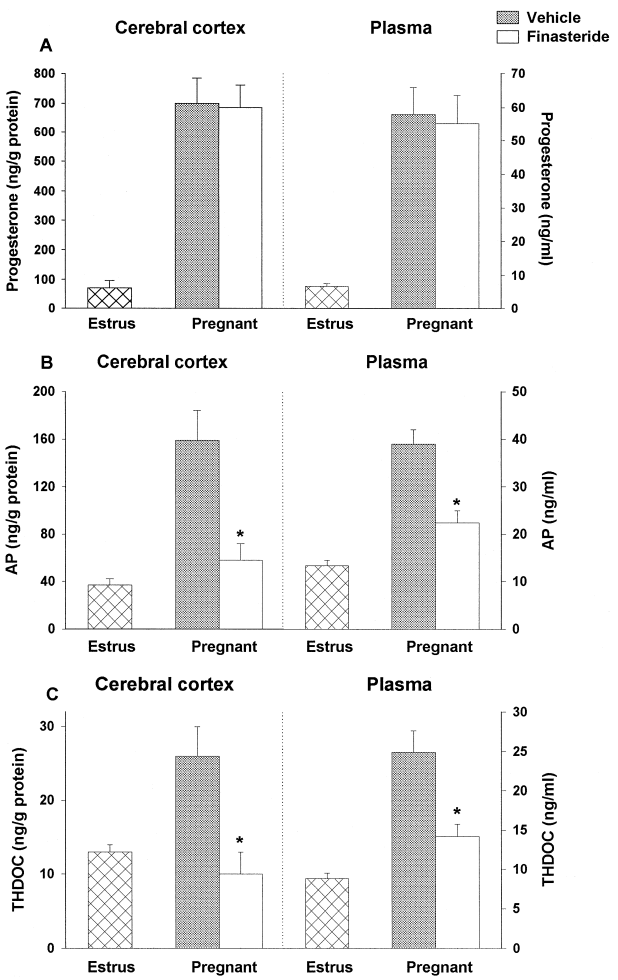

Fig. 6. Effects of subchronic treatment with finasteride on cerebrocortical and plasma concentrations of progesterone, allopregnanolone, and allotetrahydrodeoxycorticosterone in pregnant rats. Finasteride (25 mg/kg, s.c.) or vehicle was injected daily from days 12 to 18 of pregnancy and rats were killed on day 19. Data are means ± S.E.M. of values obtained from 15 rats. $^*P < 0.05$ vs. vehicle.

Table 3
Effect of subchronic treatment with finasteride on γ2L subunit mRNA in the cerebral cortex and hippocampus of estrus and pregnant rats

	γ2L mRNA (% of estrus)	
	Cerebral cortex	Hippocampus
Estrus	100 ± 2.3	100 ± 3.0
Pregnant	72 ± 4.3[a]	73 ± 4.0[a]
Pregnant + finasteride	91 ± 6.0	101 ± 6.0

Finasteride (25 mg/kg, s.c.) or vehicle was injected daily from days 12 to 18 of pregnancy, and rats were killed on day 19. Data are means ± S.E.M. of 10–14 animals for each group.
[a] $P < 0.01$ vs. corresponding estrus value.

4. Discussion

We have shown that the changes in the density and subunit expression of $GABA_A$ receptors that occur in the rat brain during pregnancy and after delivery are related to the changes in plasma and cortical concentrations of progesterone neuroactive steroid derivatives. Measurements of [^{3}H]GABA, [^{3}H]flunitrazepam and [^{35}S]TBPS bindings to cerebrocortical membranes revealed a progressive increase in the density of recognition sites associated with the $GABA_A$ receptor complex during pregnancy. The maximal increase in receptor density was apparent on day 19 of pregnancy and was followed by a marked decrease immediately before delivery (day 21 of pregnancy) and a further decrease apparent 2 days postpartum. These changes were accompanied by a reduction in the apparent affinities of GABA, benzodiazepine and TBPS recognition sites during pregnancy, although the K_D values after delivery did not differ significantly from those apparent during estrus. These data are consistent with previously described (Majewska, 1992) changes in [^{3}H]muscimol binding in the rat forebrain during pregnancy and in the postpartum period, while are in contrast with more recent data (Weizman et al., 1997) showing no modification in $GABA_A$ and central benzodiazepine receptors in the cerebral cortex during pregnancy.

The changes in $GABA_A$ receptor density during pregnancy and after delivery are associated with alterations in receptor function. Thus, we have previously reported (Concas et al., 1998; Follesa et al., 1998) a lower sensitivity of the GABA-coupled chloride channel to the action of muscimol during the last week of pregnancy. On the contrary, a marked enhancement in the efficacy of muscimol in stimulating $^{36}Cl^-$ uptake was apparent after delivery. These data may reflect to some extent physiological changes in the activity of the chloride channel during pregnancy and after delivery, respectively. Accordingly, the abilities of diazepam and allopregnanolone to potentiate the muscimol response on $^{36}Cl^-$ uptake was decreased during pregnancy and increased after delivery (Follesa et al., 1998).

The time course of the changes in the brain concentrations of allopregnanolone and allotetrahydrodeoxycorticosterone, the most potent and efficacious positive endogenous modulators of $GABA_A$ receptors (Majewska, 1992; Lambert et al., 1995), during pregnancy are similar to those of the changes in the density and activity of $GABA_A$ receptors. Thus, the marked increase in the circulating concentration of progesterone during pregnancy is paralleled by increases in the concentrations of allopregnanolone and allotetrahydrodeoxycorticosterone in both plasma and brain. The cortical neurosteroid levels peaked on day 19 of pregnancy, coincident with the maximal increase in the density of GABA, benzodiazepine and TBPS recognition sites (Fig. 3) and the maximal reduction in the chloride channel function (Follesa et al., 1998). In contrast, on day 21 of gestation, the marked decrease in plasma and cortical concentrations of steroids that precedes delivery was paralleled by a restoration of the control density and function of $GABA_A$ receptors.

On the basis of these data, we propose that the changes in cortical $GABA_A$ receptor density and activity during pregnancy are functionally related to the parallel changes in the brain concentration of neuroactive steroids. Several studies have indicated that long-term treatment with neuroactive steroids can affect $GABA_A$ receptors. Progesterone has been shown to produce a region-specific up-regulation of [^{3}H]muscimol and [^{3}H]flunitrazepam binding sites in the brain (Gavish et al., 1987; Canonaco et al., 1989). Furthermore, both in vivo and in vitro experiments with cultured mammalian cortical neurons have shown that persistent exposure to neurosteroids reduces both the ability of GABA to stimulate $^{36}Cl^-$ uptake as well as the efficacy of benzodiazepines and neurosteroids (Costa et al., 1995; Yu and Ticku, 1995).

Similarly, the marked decrease in the plasma and brain concentrations of progesterone and its neuroactive metabolites immediately before delivery may underlie the sudden restoration of $GABA_A$ receptor density and function apparent at this time as well as the further changes that occur after delivery. Steroid hormone deprivation elicited by ovariectomy or ovariectomy plus adrenalectomy results in a decrease in the maximal number of GABA binding sites in the rat brain (Jussofie et al., 1995). Thus, the increase in brain neurosteroids concentrations during the first 19 days of pregnancy can be compared to the long-term administration of high doses of neurosteroids, whereas the marked fall in the concentrations of progesterone and its metabolites apparent at the end of pregnancy and after delivery is similar to a sudden discontinuation of such treatment.

This latter conclusion is supported by the effects of finasteride inhibition of the activity of 5α-reductase, the enzyme that converts progesterone to its 5α-reduced metabolites (allopregnanolone and allotetrahydrodeoxycorticosterone), in various tissues including brain (Celotti et al., 1992). Thus, administration of finasteride to pregnant rats both reduced the increase in brain concentrations of allopregnanolone and allotetrahydrodeoxycorticosterone as well as prevented the increase in cortical $GABA_A$ receptor density and the reduction in the sensitivity of $GABA_A$ receptor to the action of muscimol that are normally apparent during pregnancy (Concas et al.; Follesa et al., 1998).

Our data indicate that changes in $GABA_A$ receptor density during pregnancy and after delivery are associated with opposite changes in receptor activity and that these alterations are functionally related to the changes in brain and plasma steroid concentrations. Thus, the consecutive decrease and increase in $GABA_A$ receptor function during these periods may reflect an adaptative mechanism (i) to counteract the increased stimulation of $GABA_A$ receptors by neurosteroids during pregnancy and (ii) to compensate for the reduced inhibitory tone when both neurosteroid concentrations and $GABA_A$ receptor density are decreased immediately before and after delivery.

Our results also demonstrate that pregnancy and delivery are accompanied by marked changes in the expression of specific $GABA_A$ receptor genes. Thus, the amount of the γ2L subunit mRNA decreased in the cerebral cortex and hippocampus during pregnancy and returned to control values around delivery. On the other hand, the α4 subunit mRNA remained unchanged during pregnancy and increased late in the postpartum period. These observations, together with the evidence that the amounts of α1, α2, α3, β1, β2, β3 and γ2S subunit mRNAs were not affected during pregnancy or after delivery, suggest that the changes in γ2L and α4 subunits are time and region specific and do not represent a generalized phenomenon. Accordingly, it as been recently shown (Fenelon and Herbison, 1996; Brussard et al., 1997) that the expression of α1 subunit mRNA increases during the course of pregnancy specifically in hypothalamic magnocellular neurons, which play a critical role in promoting parturition and lactation, whereas an increase in γ2 gene expression is apparent in the same area only after delivery. Thus, perhaps only specific neurons containing specific populations of $GABA_A$ receptors may contribute to the observed response.

The γ subunit plays a key role in conferring sensitivity to benzodiazepines (Pritchett et al., 1989). Thus, it is likely that the changes in the efficacy of benzodiazepines to enhance muscimol-stimulated $^{36}Cl^-$ uptake during pregnancy and after delivery (Follesa et al., 1998) may be related to the changes in the expression of the gene encoding for this specific subunit. Moreover, recent studies have shown that withdrawal from allopregnanolone after intermittent progesterone administration (Smith et al., 1998a) or in a model of pseudopregnancy (Smith et al., 1998b) results in an increased expression of α4 $GABA_A$ receptor subunit in the rat hippocampus. Indeed, we observed this latter change only on day 7 after delivery, a time in which the concentrations of progesterone and its metabolites are still much lower than those observed dur-

ing pregnancy. However, the long time elapsed between the dramatic fall in the steroid hormones (day 21) and the seventh day after parturition cannot be compared to a withdrawal condition. Nevertheless, pregnancy and lactation are physiological conditions associated with striking alterations in the hormonal milieu that cannot be compared to the administration of a single hormone. Thus, the possibility that the enhancement in the expression of α4 subunit mRNA is caused by mechanism different from changes in the neurosteroid concentrations cannot be ruled out.

An important problem that remains to be elucidated is whether changes in receptor plasticity are due to direct or indirect (genomic) actions of neurosteroids. The oxidation of allopregnanolone and allotetrahydrodeoxycorticosterone, which may occur in vivo, yields 5α-dihydroprogesterone and 5α-dihydrocorticosterone, both of which bind to and activate the cytosolic progesterone receptor (Rupprecht et al., 1993). However, progesterone receptor-mediated regulation by progesterone, 5α-dihydroprogesterone, and 5α-dihydrocorticosterone of $GABA_A$ receptor subunit expression and function appears unlikely. Indeed, the high concentration (250 nM) of progesterone in the brain during pregnancy, which is not affected by finasteride treatment, would be expected to preclude a genomic action of progesterone in the changes in $GABA_A$ receptors during pregnancy. Previous studies have shown that progesterone metabolites may modulate $GABA_A$ receptor gene expression by a direct action at the steroid recognition site on the $GABA_A$ receptor (Weiland and Orchinik, 1995). Thus, the finasteride-induced reversal of the increases in allopregnanolone and allotetrahydrodeoxycorticosterone concentrations and of the changes in $GABA_A$ receptor density and function (Concas et al., 1998) and in the reduction in γ2L subunit expression that occur during pregnancy suggests that these latter changes are the consequence of allopregnanolone and allotetrahydrodeoxycorticosterone physiological action at the steroid recognition site on the $GABA_A$ receptor.

In conclusion, our data demonstrate a functional correlation between changes in the cerebrocortical concentrations of neurosteroids and $GABA_A$ receptor plasticity during pregnancy and after delivery. Because $GABA_A$ receptors participate in the regulation of a variety of psychophysiological phenomena, including anxiety, sleep, depression, seizures and sexual functions, and great fluctuations in the brain content of neurosteroids are present during estrus cycle, pregnancy, menopause, stress, etc., our observations may shed light on the factors that influence the mood and behavioral changes associated with above mentioned conditions.

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