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Steroid Profiling in Brain and Plasma of Male and Pseudopregnant Female Rats after Traumatic Brain Injury: Analysis by Gas Chromatography/Mass Spectrometry

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Steroids in brain arise from the peripheral endocrine glands and local synthesis. In traumatic brain injury (TBI), the endogenous circulating hormones at the time of injury are important for neuroprotection. In particular, pseudopregnant females recover better than males from TBI. We investigated the effect of pseudopregnancy and TBI on steroid levels in plasma and in three brain regions (within, adjacent, and distal to the lesion site), 6 and 24 h after prefrontal cortex injury. The following steroids were analyzed by gas chromatography/mass spectrometry: pregnenolone, progesterone, 5α-dihydroprogesterone, 3α,5α-tetrahydroprogesterone, 3β,5α-tetrahydroprogesterone, dehydroepiandrosterone, Δ^4-androstenedione, testosterone, 5α-dihydrotestosterone, 3α,5α-tetrahydrotestosterone, 3β,5α-tetrahydrotestosterone, and 17β-estradiol. Corticosterone was assayed in plasma to account for stress in the rats. We found different steroid profiles in brain and plasma of male and pseudopregnant female rats and specific profile changes after TBI. In sham-operated pseudopregnant females, much higher levels of progesterone, 5α-dihydroprogesterone, 3α,5α-tetrahydroprogesterone, and 3β,5α-tetrahydroprogesterone were measured in both brain and plasma, compared with sham-operated males. Plasma levels of corticosterone were high in all groups, indicating that the surgeries induced acute stress. Six hours after TBI, the levels of pregnenolone, progesterone, and 5α-dihydroprogesterone increased, and those of testosterone decreased in male brain, whereas levels of 5α-dihydroprogesterone and 3β,5α-tetrahydroprogesterone increased in brain of pseudopregnant female rats. Plasma levels of 5α-dihydroprogesterone did not change after TBI, suggesting a local activation of the 5α-reduction pathway of progesterone in both male and pseudopregnant female brain. The significant increase in neurosteroid levels in the male brain after TBI is consistent with their role in neuroprotection. In pseudopregnant females, high levels of circulating progestagens may provide protection against TBI. (*Endocrinology* 148: 2505–2517, 2007)

THE NERVOUS SYSTEM is an important target of steroid hormones. The mechanisms by which these steroids exert their effects were construed as a classical endocrine mechanism involving production by endocrine glands, secretion into the bloodstream, crossing of the blood-brain barrier, and then regulation of central nervous system (CNS) functions (1–3). A further advance was the finding that androgens act in the CNS after their local conversion into estrogens by aromatization (4). Finally, the discovery of the local *de novo* synthesis of neurosteroids from cholesterol in the nervous system (5) has added paracrine and/or autocrine mechanisms to the list of ways by which steroids can regulate brain functions. The CNS can synthesize steroids as well as take them up from the blood. Thus, steroid brain concentrations are in part related to their peripheral production in endocrine organs. Figure 1 summarizes the main pathways of steroidogenesis. All steroids are derived from cholesterol. The cytochrome P450 side-chain cleavage enzyme is involved in the conversion of cholesterol to pregnenolone (PREG). PREG can be converted either to progesterone (PROG) by the 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD), or to dehydroepiandrosterone (DHEA) by the P450c17 enzyme. PROG is a key hormone that gives rise to all the other steroid hormones. Indeed, PROG can be converted to 11-deoxycorticosterone by the P450c21 enzyme and then to corticosterone by P45011β. PROG can also be metabolized to Δ^4-androstenedione by the P450c17 enzyme and then to testosterone by the 17β-hydroxysteroid dehydrogenase enzyme. Testosterone can be converted to 17β-estradiol by the aromatase. In addition, PROG and testosterone can be converted respectively to 5α-dihydroprogesterone (5α-DHPROG) and to 5α-dihydrotestosterone (5α-DHT) by the steroid 5α-reductases (two distinct isozymes 1 and 2) and then to 3α,5α-

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Abbreviations: AL, Adjacent to the lesion; CNS, central nervous system; DHEA, dehydroepiandrosterone; 5α-DHPROG, 5α-dihydroprogesterone; 5α-DHT, 5α-dihydrotestosterone; DL, distal to the lesion; GC/MS, gas chromatography coupled to mass spectrometry; 3α-HSD, 3α-hydroxysteroid oxidoreductase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 3β-HSOR, 3β-hydroxysteroid oxidoreductase; Km, apparent Michaelis constant; L, lesion site; mPR, membrane PR; PR, progesterone receptor; PREG, pregnenolone; PROG, progesterone; SIM, single ion monitoring; TBI, traumatic brain injury; 3α,5α-THPROG, 3α,5α-tetrahydroprogesterone; 3β,5α-THPROG, 3β,5α-tetrahydroprogesterone; 3α,5α-THT, 3α,5α-tetrahydrotestosterone; 3β,5α-THT, 3β,5α-tetrahydrotestosterone.

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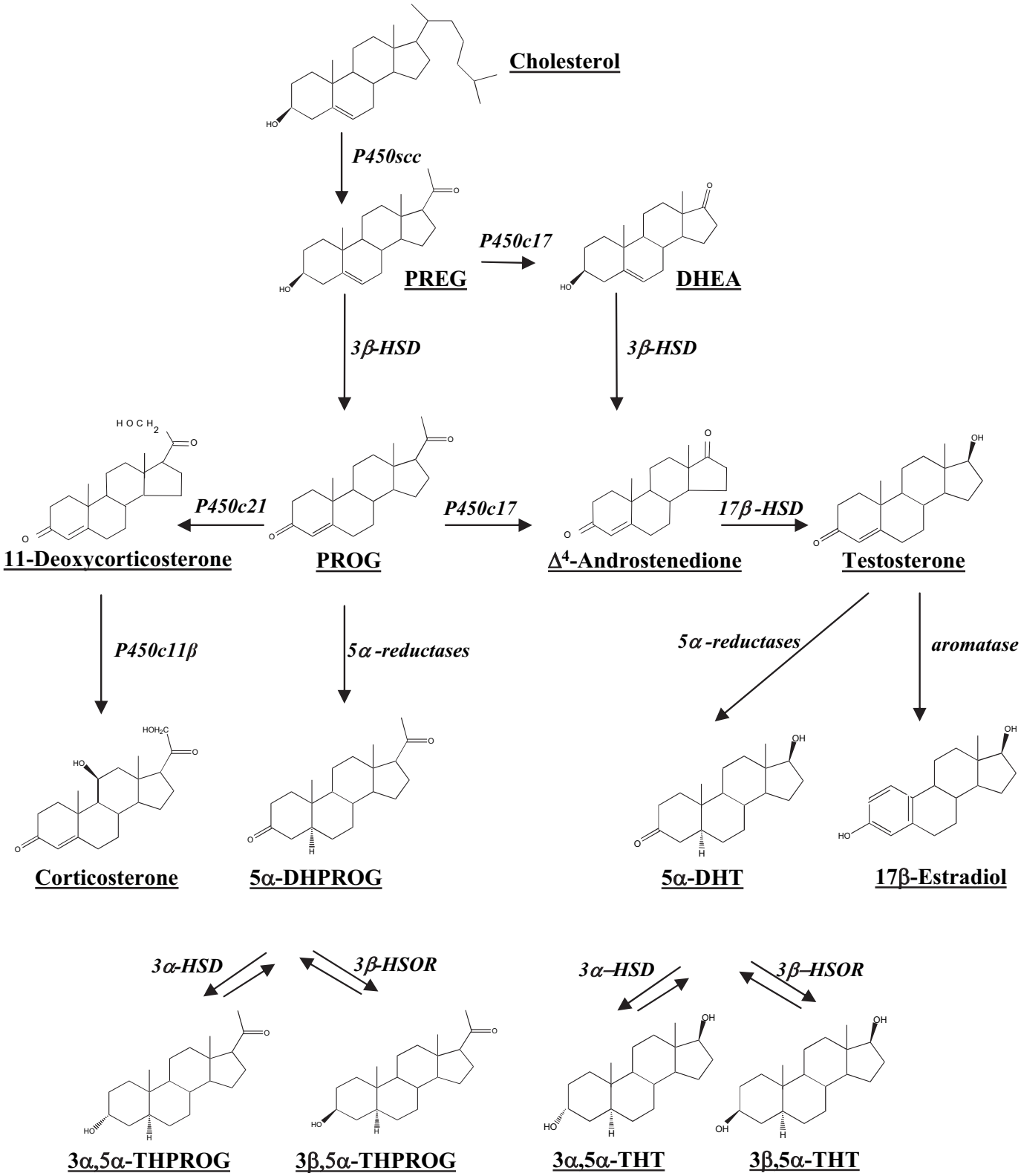


FIG. 1. Pathways of steroidogenesis. The abbreviated name of the enzyme involved for each reaction is indicated. Steroids included: 3α,5α-THPROG, 5α-DHPROG (5α-dihydroprogesterone). Enzymes included: 3α-HSD, 3β-HSD/Δ^5-Δ^4 isomerase, 3β-HSOR, 17β-HSD (17β-hydroxysteroid dehydrogenase), P450c11β (11β-hydroxylase), P450c17 (17α-hydroxylase/C17–20-lyase), P450c21 (21-hydroxylase), P450scc (cytochrome P450 side-chain cleavage).

tetrahydroprogesterone (3α,5α-THPROG) and 3α,5α-tetrahydrotestosterone (3α,5α-THT) by the 3α-hydroxysteroid oxidoreductase (3α-HSD). In addition, 5α-DHPROG and 5α-DHT can be reduced, respectively, to 3β,5α-tetrahydroprogesterone (3β,5α-THPROG) and 3β,5α-tetrahydrotestosterone (3β,5α-THT) by the 3β-hydroxysteroid oxidoreductase (3β-HSOR).

It is now clear that PROG and its reduced metabolites (5α-DHPROG and 3α,5α-THPROG), and estrogens exert a variety of neuroprotective effects and that they may be good candidates for therapeutic tools after spinal cord and brain injuries and in neurodegenerative diseases (6–12). A recent phase II clinical trial with PROG in patients with moderate to severe traumatic brain injury (TBI) revealed that 3 d of treatment with PROG could reduce mortality by more than 50% in the severely injured group (13).

Initial evidence that PROG could be used as a pharmacological agent after brain injury came from studies on gender differences in recovery, which also suggested that endogenous levels of PROG could enhance recovery of function from TBI (14). Indeed, high levels of endogenous PROG promote behavioral recovery and decrease the cytotoxic sequel of trauma. For example, compared with males, postinjury edema is lower in normal-cycling females and practically eliminated in pseudopregnant females (a hormonal state in which PROG levels remain high for about 10 d after cervical stimulation) after medial frontal cortical contusions (15). Not only does treatment with exogenous steroids decrease the response to various forms of insult, but also endogenous levels at the time of injury are important for neuroprotection (7, 8). Furthermore, the nervous system itself may adjust and adapt both steroid synthesis and steroid receptor expression after injury, as we have demonstrated for the brain and spinal cord (16, 17). One important question is the impact of the steroids either produced by peripheral steroidogenic organs or pharmacologically administered on steroid synthesis by the CNS. Castration and adrenalectomy had no effects on 3β-HSD expression in spinal cord (18) whereas estrogen treatment increased 3β-HSD expression and activity in the hypothalamus of female rats (19). Progesterone synthesis has been shown to be stimulated by estradiol in the hypothalamus and enriched astrocyte cultures (19–21). Variation in the production of PROG from peripheral glands could influence levels of brain PROG and its metabolites. Indeed, PROG originating in the periphery is taken up by the brain in considerable amounts (22), and the conversion of PROG to its neuroactive metabolites takes place within the brain (12, 23–25). Apart from being a substrate for this metabolism, PROG exerts its own hormonal effects through specific receptors.

Because pseudopregnant females have better outcomes than males and steroids have neuroprotective effects after TBI, we examined the local modifications of steroid levels in males and pseudopregnant females after TBI to gain insight into the hormonal determination of neuroprotection. First, we compared steroid levels between male and pseudopregnant female rats to evaluate how variations in plasma concentrations of steroids are reflected in brain. Second, we investigated the effect of TBI on steroid levels in plasma and three brain regions (within, adjacent to, and distal to the lesion site) to evaluate how hormonal state at the time of injury could affect the response of the nervous tissue to injury.

Materials and Methods

Subjects

Adult male and female Sprague Dawley rats approximately 90 d of age at the beginning of the experiment were housed individually and kept on a reverse light-dark cycle (0800–2000 h). All procedures concerning animal care and use were carried out in accordance with the European Community Council Directive (86/609/EEC) and conformed to guidelines set forth in the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996), and were approved by the Emory University Institutional Animal Care and Use Committee, protocol 098-2001.

Pseudopregnancy induction

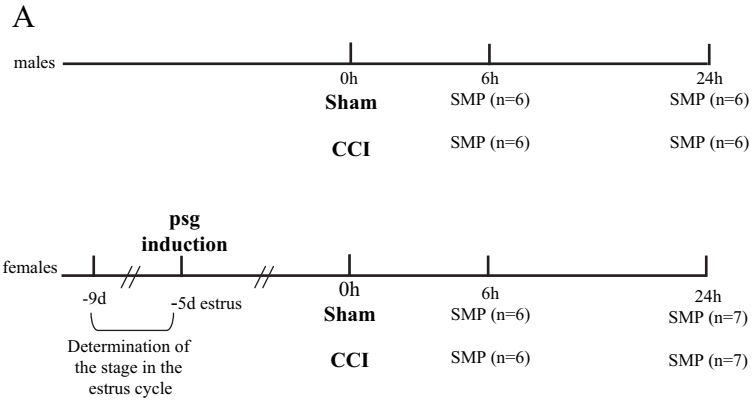
Vaginal lavages were used to determine the time of pseudopregnancy induction and confirm that rats were pseudopregnant at the time of injury. Lavages were collected and analyzed daily beginning 1 d after the rat's arrival in the laboratory and continued through the day of surgery. The epithelium of the vagina was flushed with saline solution (0.9%) using a blunt-end plastic pipette and then dispersing the pipette's contents onto a clean microscope slide. The samples were analyzed under a light microscope and the cell types present (leukocytes, nucleated epithelial cells, and/or cornified epithelial cells) were recorded. The stage in the estrous cycle (metestrus, diestrus, proestrus, or estrus) at the time of the lavage was determined based on the presence or absence of these cell types. Pseudopregnancy was induced 5 d before surgery by stimulating the cervix mechanically using the soft, rubber-tipped plunger of a plastic 1-cc syringe for 1 min when females were in estrus and exhibiting cornified cells (15).

Animals and experimental injury model (Fig. 2A)

Eight groups of rats were prepared (n = 6 or 7/group): four conditions (sham-operated males, males with TBI, sham-operated pseudopregnant females, and pseudopregnant females with TBI) and two times of analysis (6 and 24 h after surgery) were chosen to profile steroid levels in brain and plasma. The controlled cortical impact injury for the females was done on their fifth day of pseudopregnancy, when they were exhibiting abundant leukocytes. Surgery for an equal number of males was done when females were to be injured. Bilateral contusions of the medial prefrontal cortex of male and pseudopregnant female rats were made with a pneumatic impactor device (see Ref. 26 for details). Briefly, animals were anesthetized with ketamine/xylazine (90 mg/kg per 10 mg/kg) and placed in a stereotaxic apparatus equipped with a homeothermic blanket system to maintain body core temperature at 37 C. A midline incision was made in the scalp, the fascia was retracted to expose the cranium, and a craniectomy (6 mm diameter) was made over the midline of the prefrontal cortex with its center 3.0 mm anteroposterior to bregma. After removal of the bone, the tip of the impactor (5 mm diameter) was moved to +3.0 mm anteroposterior, 0.0 mm (from bregma), and checked for adequate clearance. Trauma was produced by activating the piston with compressed air to impact −2.0 mm dorsoventral (from dura) at a velocity of 2.25 m/sec with a brain contact time of 0.5 sec. After the contusion injury, the wound cavity was cleaned and bleeding stopped before scalps were sutured. Sham-operated animals sustained the anesthesia, incision, and suture of the scalp.

Samples for steroid determination

Rats were killed by decapitation 6 and 24 h after surgery. For each rat, four samples were collected. A sample from cardiac blood was taken and the brain dissected out of the skull on a bed of crushed ice. Three brain regions were collected from each brain (Fig. 2B): contused tissue [lesion site (L)], bilateral samples adjacent to the lesion [adjacent to the lesion (AL)], and bilateral samples from posterior cortex [distal to the lesion (DL)]. Brain samples were weighed, frozen on dry ice, and stored at −80 C until gas chromatography coupled to mass spectrometry (GC/MS) analysis.



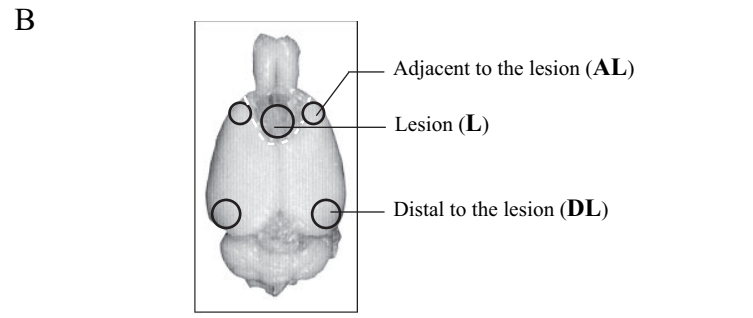


FIG. 2. Experimental groups and samples used for steroid analysis by GC/MS. A, Summary of experimental groups and time course of the pseudopregnancy induction and injury. Eight groups were used: sham-operated males, males with TBI, sham-operated pseudopregnant females, and pseudopregnant females with TBI; with two times of analysis, 6 and 24 h after injury. CCI, Controlled cortical impact; n, number of animals per group; psg, pseudopregnancy; SMP, sampling. B, Location of the samples taken for steroid determination. In addition to the plasma, three brain regions were collected from each animal: contused tissue (L), bilateral samples adjacent to the lesion (AL), and bilateral samples from posterior cortex (DL).

Measurement of steroid levels by GC/MS

PREG, PROG, 5α-DHPROG, 3α,5α-THPROG, 3β,5α-THPROG, Δ^4-androstenedione, testosterone, 5α-DHT, 3α,5α-THT, 3β,5α-THT, DHEA, and 17β-estradiol levels were determined by GC/MS according to the protocol described by Liere *et al.* (27) with minor modifications. Briefly, steroids were extracted from individual brain regions (the weight range was 93–450 mg of tissue) and plasmas (1 ml) by adding 10 volumes of methanol. Corticosterone was measured only in plasmas 6 and 24 h after injury to document the stress state of the animals. The internal standards epietiocholanolone (for PREG, 3α,5α-THPROG, 3β,5α-THPROG, Δ^4-androstenedione, testosterone, 5α-DHT, 3α,5α-THT, 3β,5α-THT, DHEA, and 17β-estradiol), 19-nor PROG (for PROG), $^2H_6$-5α-DHPROG (for 5α-DHPROG), and $^2H_8$-corticosterone (for corticosterone) were introduced into the extract for steroid quantification. Samples were purified and fractionated by solid-phase extraction with the recycling procedure (28) and HPLC as previously described (16). Three fractions were collected from the HPLC system: 5α-DHPROG and $^2H_6$-5α-DHPROG were eluted in the first HPLC fraction (3–10 min) and were silylated with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide/NH_4I/dithioerythritol (1000:2:5 vol/vol/vol) for 15 min at 70 C. The second fraction (10–31 min) contained PREG, PROG, 3α,5α-THPROG, 3β,5α-THPROG, Δ^4-androstenedione, testosterone, 5α-DHT, 3α,5α-THT, 3β,5α-THT, DHEA, 17β-estradiol, epietiocholanolone, and 19-nor PROG. Corticosterone and its internal standard $^2H_8$-corticosterone were eluted in the third HPLC fraction (31–45 min). These two latter fractions were derivatized with heptafluorobutyric anhydride in anhydrous acetone for 30 min at 20 C. All the fractions were dried under a stream of N_2 and resuspended in hexane for GC/MS analysis.

Calibration and biological samples were analyzed by GC/MS with an AS 2000 autosampler (Carlo Erba, Milan, Italy). The Trace^{GC} gas chromatograph (Carlo Erba) is coupled with an Automass Solo mass spectrometer (Thermo Electron, Les Ulis, France). Injection was performed in the splitless mode at 250 C (1 min of splitless time), and the temperature of the gas chromatograph oven was initially maintained at 50 C for 1 min and then ramped between 50 and 340 C at 20 C/min. The helium carrier gas flow was maintained constant at 1 ml/min during the analysis. The transfer line and ionization chamber temperatures were 300 and 180 C, respectively. Electron impact ionization was used for mass spectrometry with an ionization energy of 70 eV. Identification of each steroid was supported by its retention time and its two diagnostic ions (see Table 1). Quantification was performed in single ion monitoring (SIM) mode according to the major diagnostic ion, called quantification ion, and to the retention time of each derivatized steroid.

The analytical protocol has been previously validated (16) for PREG, PROG, and their reduced metabolites. The same experimental procedure was applied to validate the quantification of DHEA, testosterone, 5α-DHT, 3α,5α-THT, 3β,5α-THT, Δ^4-androstenedione, 17β-estradiol, and corticosterone. Only testosterone could be detected in all the aliquots of extracts from an adult male brain (10, 20, 50, 100, 200, and 300 mg). DHEA and 3α,5α-THT were detected only in 300 mg of brain extract. In male rat plasma, testosterone and 3α,5α-THT were detected in all the plasma extracts (50, 100, 200, 500, 1000 μl) and Δ^4-androstenedione and 5α-DHT could be detected from 1 ml of plasma. The overall results of validation are summarized in Table 2 in terms of basal values, coefficient of variation, coefficient of correlation, and limit of detection in plasma (1 ml) and brain (100 mg).

Statistical analysis

All data were analyzed by a commercially available program (GraphPad Prism 3.0; GraphPad Inc., San Diego, CA). For brain samples, data were processed using two-way ANOVA (treatment × region), followed by Bonferroni *post hoc* test. For plasma samples, Student's *t* test was used. Statistical significance was noted when the probability of type I error was less than 0.05.

Results

Steroid profiles in brain regions and plasma of sham-operated male and pseudopregnant female rats

The different steroids were found in a very wide range of concentrations in brain and plasma of sham-operated male and pseudopregnant female rats (Figs. 3–5, *white bars*). In sham-operated males, the decreasing order of mean concentrations was: PREG > PROG ≈ testosterone > 5α-DHPROG = 5α-DHT > 3α,5α-THT = Δ^4-androstenedione in

TABLE 1. GC/MS parameters used for steroid identifications in SIM detection

Steroids (molecular weight)	Derivatized steroids (molecular weight)	Retention time (min)	Diagnostic ions (m/z)
Screened steroids			
PREG (316)	PREG-3-HFB (512)	15.85	283 and **298**
PROG (314)	PROG-3-HFB (510)	15.80	495 and **510**
5α-DHPROG (316)	5α-DHPROG-3,20-TMS_2 (460)	16.30	445 and **460**
3α,5α-THPROG (318)	3α,5α-THPROG-3-HFB (514)	15.26	496 and **514**
3β,5α-THPROG (318)	3β,5α-THPROG-3-HFB (514)	16.00	496 and **514**
Corticosterone (346)	Δ^{9-11} Corticosterone-3,21-HFB_2 (720)	15.94	**705** and 720
DHEA (288)	DHEA-3-HFB (484)	14.76	255 and **270**
Testosterone (288)	Testosterone-3,17-HFB_2 (680)	12.93	665 and **680**
5α-DHT (290)	5α-DHT-17-HFB (486)	15.28	414 and **486**
3α,5α-THT (292)	3α,5α-THT-3,17-HFB_2 (684)	12.38	455 and **470**
3β,5α-THT (292)	3β,5α-THT-3,17-HFB_2 (684)	13.09	455 and **470**
Δ^4-Androstenedione (286)	Δ^4-Androstenedione-3-HFB (482)	14.78	467 and **482**
17β-Estradiol (272)	17β-Estradiol-3,17-HFB_2 (664)	13.24	451 and **664**
Internal standards			
Epietiocholanolone (290)	Epietiocholanolone-3-HFB (486)	14.21	442 and **486**
19 Nor-PROG (300)	19 Nor-PROG-3-HFB (496)	15.74	481 and **496**
$^2H_6$-5α-DHPROG (322)	$^2H_6$-5α-DHPROG-3,20-TMS_2 (466)	16.28	**451** and 466
$^2H_8$-corticosterone (354)	Δ^{9-11}- $^2H_8$-corticosterone-3,21-HFB_2 (728)	15.90	**709–713** and 724–728

The diagnostic ions were used together with the retention time for identification in the SIM mode of the derivatized steroids. The diagnostic ions in *bold* were used for quantification. HFB, Heptafluorobutyrate; TMS, trimethylsilyl.

brain and PROG > PREG = testosterone > 5α-DHPROG > Δ^4-androstenedione ≈ 3α,5α-THT > 5α-DHT in plasma (Figs. 3 and 4, *white bars*). In sham-operated pseudopregnant females, the decreasing order of concentrations was: PROG ≈ 5α-DHPROG > PREG ≥ 3α,5α-THPROG > 3β,5α-THPROG in brain and PROG > 5α-DHPROG ≈ 3α,5α-THPROG ≥ PREG > 3β,5α-THPROG in plasma (Fig. 5, *white bars*). In both plasma and brain, the concentrations of PROG and its metabolites were much higher in pseudopregnant females than males. The levels of 5α-DHPROG in the pseudopregnant female rats were about 10 times higher in brain, compared with plasma, suggesting that most of the reduction of PROG takes place in the brain itself. Furthermore, we calculated the ratio of reduced PROG metabolites (5α-DHPROG, 3α,5α-THPROG, and 3β,5α-THPROG) to PROG in brain and plasma to provide an index of the conversion of the

TABLE 2. Validation of steroid measurements in male rat brain and plasma by GC/MS

Steroids	Concentrations (ng/g or ng/ml ± SEM)	Coefficient of variation (%)	Coefficient of correlation	Limit of detection (ng/g or ng/ml)
Brain				
PREG	4.91 ± 0.27	13	0.9967	0.25
PROG	0.36 ± 0.01	4	0.9961	0.10
5α-DHPROG	0.75 ± 0.10	10		0.25
3α,5α-THPROG	0.41 ± 0.05	21		1.00
3β,5α-THPROG	ND			0.25
Corticosterone	50.1 ± 2.2	10	0.9951	2.00
DHEA	0.19 ± 0.01	18		0.50
Testosterone	1.42 ± 0.09	9	0.9952	0.05
5α-DHT	ND			0.10
3α,5α-THT	0.03 ± 0.003	20		0.02
3β,5α-THT	ND			0.02
Δ^4-Androstenedione	ND			0.01
17β-Estradiol	ND			0.05
Plasma				
PREG	0.58 ± 0.03	10	0.9981	0.05
PROG	2.17 ± 0.07	7	0.9996	0.01
5α-DHPROG	ND			0.05
3α,5α-THPROG	ND			0.10
3β,5α-THPROG	ND			0.02
Corticosterone	54.4 ± 1.35	5	0.9948	0.20
DHEA	ND			0.05
Testosterone	1.24 ± 0.05	10	0.9935	0.005
5α-DHT	0.03 ± 0.01	19		0.02
3α,5α-THT	0.16 ± 0.01	5	0.9998	0.002
3β,5α-THT	ND			0.002
Δ^4-Androstenedione	0.10 ± 0.01	17	0.9942	0.001
17β-Estradiol	ND			0.005

This table summarized the overall results of validation in terms of basal values, coefficient of variation, coefficient of correlation, and limit of detection in plasma (1 ml) and brain (100 mg). The coefficients of correlation confirm the linear relationship between the rat brain weight (from 10 to 300 mg) or plasma volume (from 50 to 1000 μl) and the endogenous amounts of steroids. ND, Not detected.

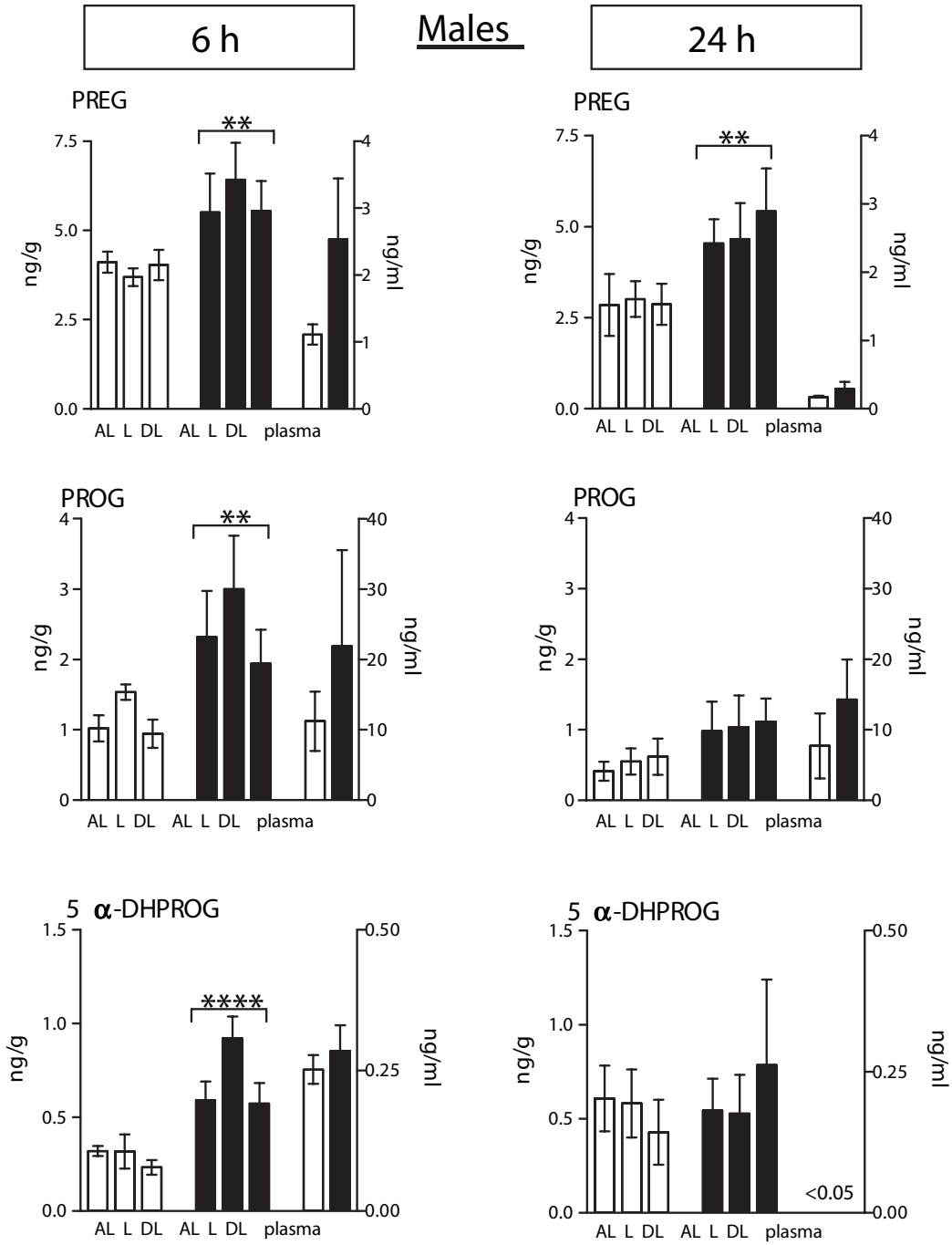


FIG. 3. Steroid levels in brain and plasma of adult male rats 6 and 24 h after sham operation or TBI. Concentrations of PREG, PROG, and 5α-DHPROG in three brain regions (L, AL, DL) and plasma. *White bars*, Levels for sham-operated rats; *black bars*, effect of TBI. Data represent mean ± SEM of six rats. Statistical analysis: two-way ANOVA for brain; Student's *t* test for plasma samples. **, $P < 0.01$; ****, $P < 0.0001$.

parent hormone PROG to its reduced metabolites. This ratio was 5–8 times higher in brain than plasma, further supporting the hypothesis that the reduced metabolites of PROG detected in pseudopregnant female rat brain are very likely locally synthesized and that only a minor part is derived from the circulation. Whereas testosterone, its precursor, and its 5α-reduced metabolites were detectable in male brain and plasma, no trace of these androgens was detected in pseudopregnant female samples. 17β-estradiol levels were below the limit of detection in brain and plasma of male and pseudopregnant female rats.

Effects of TBI on steroid levels in plasma and brain of male and pseudopregnant female rats: analysis by GC/MS 6 and 24 h after injury

Males (Figs. 3 and 4). Two-way ANOVA (treatment × region) revealed a significant effect of the lesion on levels of

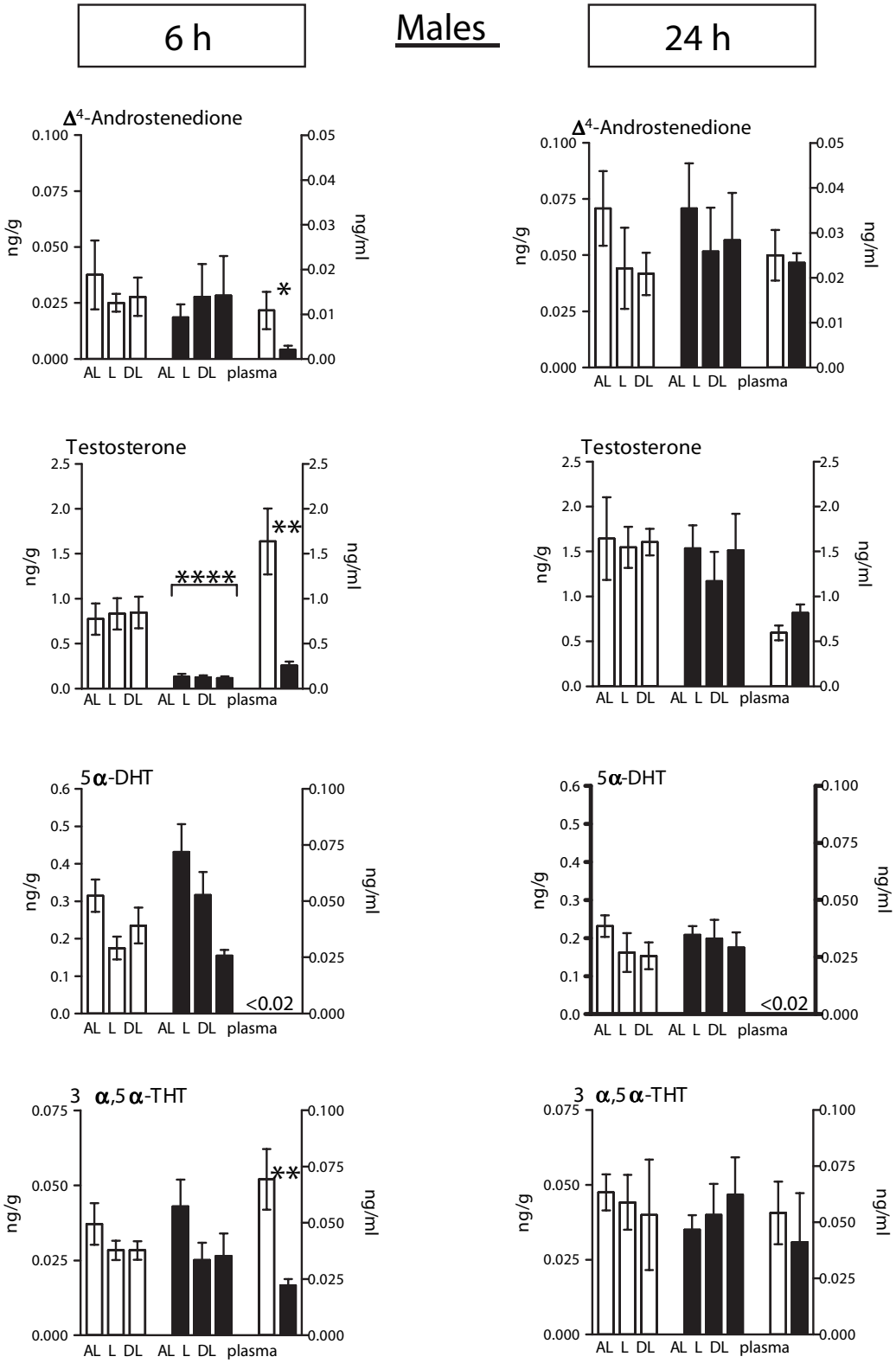


FIG. 4. Steroid levels in brain and plasma of adult male rats 6 and 24 h after sham operation or TBI. Concentrations of Δ^4-androstenedione, testosterone, 5α-DHT, and 3α,5α-THT in three brain regions (L, AL, DL) and plasma. *White bars*, Levels for sham-operated rats; *black bars*, effect of TBI. Data represent mean ± SEM of six rats. Statistical analysis: two-way ANOVA for brain; Student's *t* test for plasma samples. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

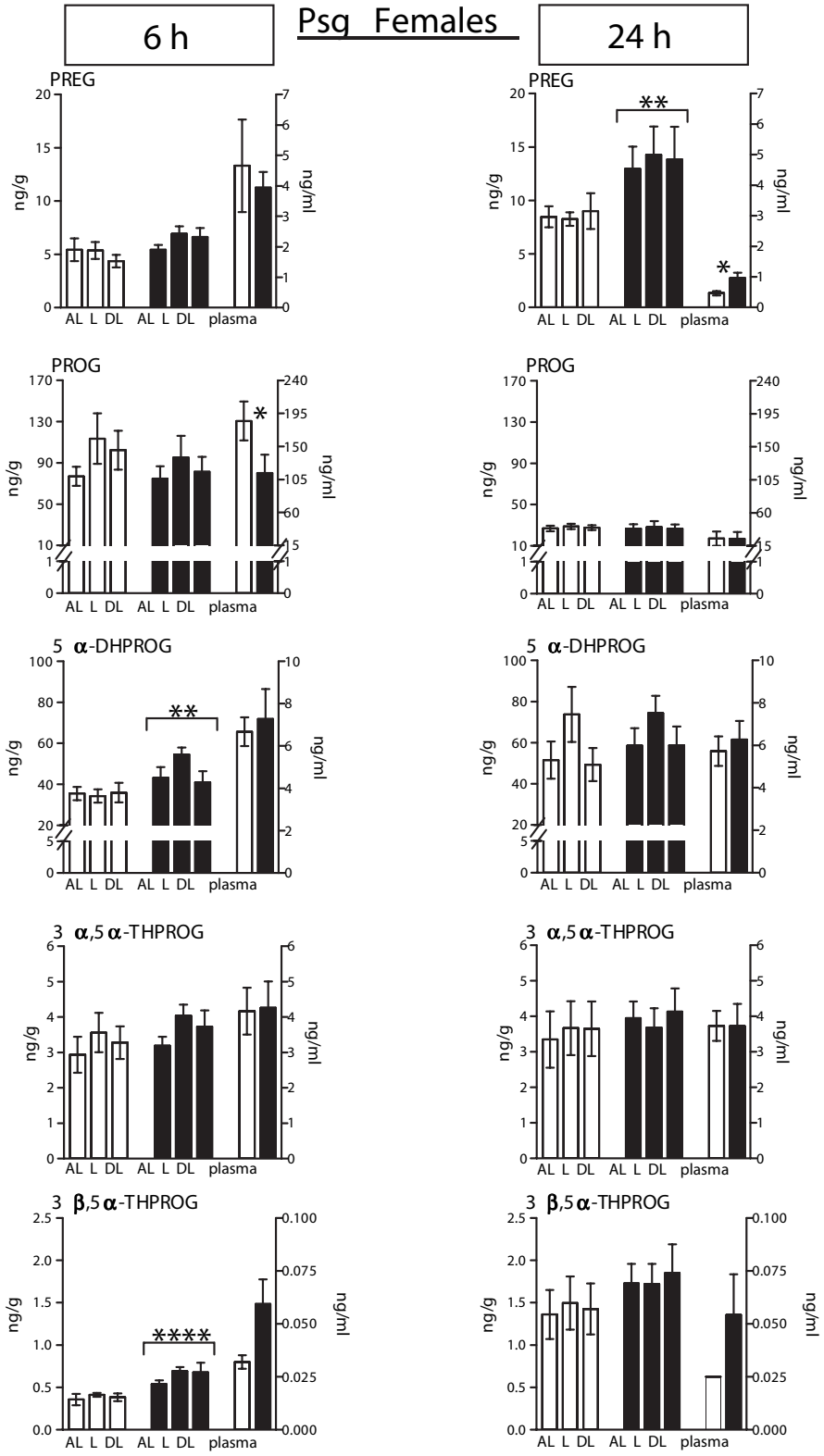


FIG. 5. Steroid levels in brain and plasma of pseudopregnant (Psg) female rats 6 and 24 h after sham operation or TBI. Concentrations of PREG, PROG, 5α-DHPROG, 3α,5α-THPROG, and 3β,5α-THPROG in three brain regions (L, AL, DL) and plasma. *White bars*, Levels for sham-operated rats; *black bars*, effect of TBI. Data represent mean ± SEM of six to seven rats. Statistical analysis: two-way ANOVA and Student's *t* test for brain samples; Student's *t* test for plasma samples. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

PREG ($F_{1,29} = 10.36$; $P < 0.01$), PROG ($F_{1,28} = 10.47$; $P < 0.01$), 5α-DHPROG ($F_{1,30} = 31.86$; $P < 0.0001$), and testosterone ($F_{1,29} = 43.49$; $P < 0.0001$) in brain 6 h after TBI. The subsequent Bonferroni *post hoc* test showed that the levels of PREG increased in L (+75%, $P < 0.05$). The levels of PROG tended to increase in the different brain regions, but the differences were not statistically significant. The levels of 5α-DHPROG increased in L (+189%, $P < 0.001$) and the posterior cortex DL (+145%, $P < 0.05$). Changes in plasma levels of PREG, PROG, and 5α-DHPROG were not statis-

tically significant. The levels of 3α,5α-THPROG and 3β,5α-THPROG were still below the threshold of detection in both brain (<1.0 and 0.25 ng/g, respectively) and plasma (<1.0 and 0.02 ng/ml, respectively). Testosterone levels decreased dramatically in the lesion site (−82%, $P < 0.01$), in the tissue AL (−85%, $P < 0.01$), posterior cortex DL (−86%, $P < 0.001$), and plasma (−84%, $P < 0.01$). No change in brain levels of Δ^4-androstenedione, 5α-DHT, or 3α,5α-THT was observed in brain samples. However, Δ^4-androstenedione and 3α,5α-THT levels decreased significantly in plasma (−85%, $P < 0.05$ and −68%, $P < 0.01$, respectively). Twenty-four hours after TBI, the differences in steroid levels between sham-operated and injured rats were not significant, except for PREG levels, which are increased in the brain ($F_{1,30}$ = 8,51; $P < 0.01$).

Pseudopregnant females (Fig. 5). Two-way ANOVA (treatment × region) revealed a significant effect of the lesion on the levels of 5α-DHPROG ($F_{1,30} = 9.73$; $P < 0.01$) and 3β,5α-THPROG ($F_{1,29} = 22.72$; $P < 0.0001$) in brain 6 h after TBI. The subsequent Bonferroni *post hoc* test for brain samples showed that the levels of 5α-DHPROG increased in the L (+59%, $P <$ 0.01). The levels of 3β,5α-THPROG increased in the L (+67%, $P < 0.05$) and posterior cortex DL (+77%, $P < 0.01$). The levels of screened androgens and 17β-estradiol were below the threshold of detection. Plasma levels of all the measured steroids did not change, except for the levels of PROG, which decreased (−41.5%, $P < 0.05$). Twenty-four hours after TBI, the differences in steroid levels between sham-operated and injured rats were not significant, except for PREG levels, which are increased in the brain ($F_{1,36}$ = 9,52; $P < 0.01$) and plasma (+105%, $P < 0.05$).

Corticosterone levels (Fig. 6). The levels of corticosterone were measured in plasma of sham-operated and injured male and pseudopregnant female rats 6 and 24 h after TBI to evaluate the extent of stress induced by the surgery. High levels of corticosterone were detected in plasma of all groups. These levels were higher in pseudopregnant females than males. In addition, these levels were higher after TBI, particularly in male rats 6 h after TBI (+105%, $P < 0.05$).

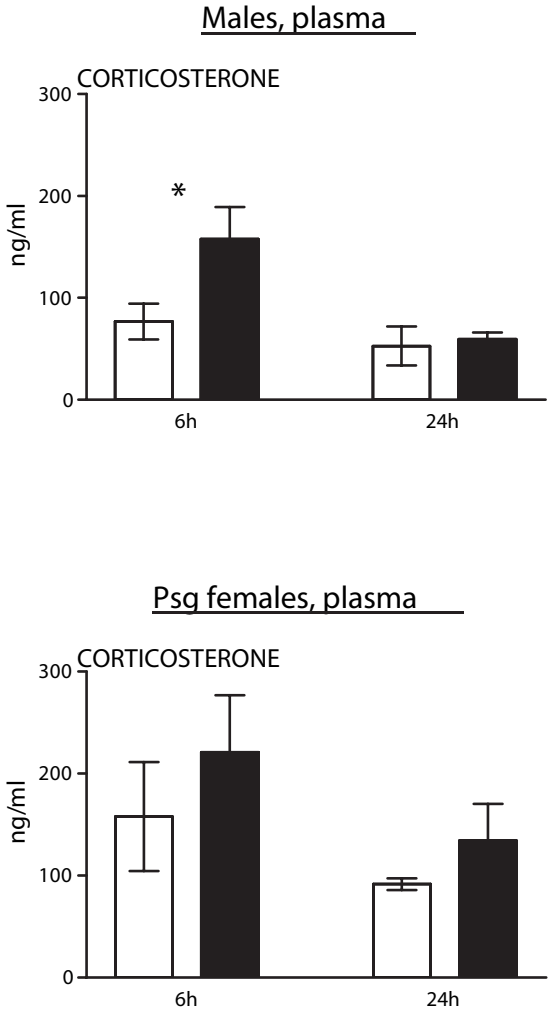


FIG. 6. Corticosterone levels in plasma of male and pseudopregnant (Psg) female rats 6 and 24 h after sham operation or TBI. *White bars*, Levels for sham-operated rats; *black bars*, effect of TBI. Data represent mean ± SEM of six to seven rats. Statistical analysis: Student's *t* test. *, $P < 0.05$.

Discussion

Our results showed different steroid profiles in brain and plasma of male and pseudopregnant female rats and transitory-specific changes after TBI. Steroids in brain have profound physiological effects that depend on their concentrations in the target tissues, their metabolism, and the distribution of receptors they activate or modulate. Because the different steroids are metabolically linked, changes in the endogenous levels of specific steroids may reflect *in vivo* regulation of their biosynthesis and metabolism, which in turn may provide insights into their physiological roles.

Sham-operated rats: comparison between males and pseudopregnant females

In this study, very high levels of PROG were measured in the brain of pseudopregnant females, compared with males, reflecting their respective levels in plasma. Indeed, the brain levels of PROG in pseudopregnant females were 47–100 times higher than those measured in males. Thus the concentration of PROG in rat brain is related to endocrine status in males and females, indicating that plasma levels determine the uptake of PROG into the brain. The concentration of PROG and its reduced metabolites in rat and human brain have been reported to be related to endocrinological status. In female rat brain, the levels of PROG, 5α-DHPROG, and 3α,5α-THPROG increased during pregnancy (29). In human female brain, the concentrations of the three steroids were significantly higher in the luteal phase, compared with postmenopausal controls. The correlation between serum and brain tissue concentrations indicates that the serum levels are directly related to the uptake of PROG in the brain (30). Because circulating PROG levels are high in pseudopregnant females, the final brain levels represent a high amount of substrate for the 5α-reductases, leading to increased levels of 5α-DHPROG, which in turn is a substrate of 3α-HSD and 3β-HSOR. Thus, detectable levels of 3α,5α-THPROG and 3β,5α-THPROG were found in brain regions of pseudopreg-

nant females, whereas the two PROG metabolites were below detection limit in brain regions of the sham-operated males. Cheney *et al.* (31) reported low levels of 3α,5α-THPROG in male rat brain (0.9 ng/g), and more recently Ebner *et al.* (32) reported very low levels of 3α,5α-THPROG (0.35 ng/g) and 3β,5α-THPROG (0.13 ng/g) in male rat brain.

The difference between our results and these reports could be due to a difference in the sensitivity of the techniques, the size of the samples used for measurements, or regional heterogeneity in the brain content of these PROG metabolites. Indeed, in the above cited studies, measurements were done in whole brain, whereas we measured steroids in three discrete, specific regions as indicated in Fig. 2. Furthermore, we performed measurements in sham-operated male rats, whereas they performed them in control rats. In any case, the levels of 3α,5α-THPROG and 3β,5α-THPROG in sham-operated pseudopregnant females brain were higher than those in sham-operated male rats (the present study) and control male rat brain (31, 32). The 3β,5α-THPROG measured in plasma and brain of pseudopregnant female rats could have arisen directly from 5α-DHPROG or through epimerization of 3α,5α-THPROG (33).

Comparison of PROG and 5α-DHPROG levels in sham-operated pseudopregnant females at 6 and 24 h after surgery corresponding to the fifth and sixth day of pseudopregnancy suggests that the 5α-reduction is an active metabolic pathway in the pseudopregnant female brain. Indeed, on the fifth day of pseudopregnancy (6 h after TBI), PROG levels in the brain were at least two times higher than those of 5α-DHPROG, whereas they were lower on the sixth day of pseudopregnancy (24 h after TBI).

We noticed that the measured values of PROG in males were relatively high in rat plasma (8–10 ng/ml for sham-operated animals), compared with normal levels, which are generally around 1–2 ng/ml (16, 27, 34). This increase in PROG levels could be due to a stress-protective reaction in response to the sham operation. The most likely hypothesis is that surgery activates the hypothalamo-pituitary-adrenal axis and stimulates the secretion of corticosterone, the final metabolite of PREG, PROG, and 11-deoxycorticosterone. Indeed, the measured levels of corticosterone in sham-operated male rats (76.8 and 52.8 ng/ml 6 and 24 h after the sham operation) indicated that the animals were stressed. Mass spectrometric studies have shown that the corticosterone levels of unstressed rats ranged from 4 to 12 ng/ml (35) and 17.1 ng/ml (36) and from 70 to 300 ng/ml for acute or chronic stress. The decrease in corticosterone levels and also in PREG, PROG, and 5α-DHPROG in plasma of sham-operated male rats 24 h after the surgery, compared with 6 h, is consistent with the acute stress hypothesis. The same tendency is found in pseudopregnant female rats for corticosterone, PREG, and PROG levels in plasma. Previous studies have shown that various types of stress lead to an increase in the brain levels of 3α,5α-THPROG and/or its precursors, PREG, PROG, and 5α-DHPROG (37–40).

Levels of Δ^4-androstenedione and testosterone in brain of sham-operated male rats were slightly higher at 24 than at 6 h after surgery. The relatively low levels at 6 h may be due to the stress in response to the sham operation, which is also attested by the increased levels of PROG and corticosterone at this time point.

Predictably, 17β-estradiol levels in brain and plasma of male and pseudopregnant female rats were below detectable limits of the technique. Indeed, using liquid chromatography/tandem mass spectrometry, 17β-estradiol could not be detected in serum and brain of male and female mice (41). In the pseudopregnant female rats, the expected concentration of 17β-estradiol in plasma are very low. The levels, measured by RIA, were within the range of those of metestrus (about 5–7 pg/ml plasma, which is the limit of detection of our technique) (42–44).

At the time of injury, there are differences between males and pseudopregnant females in the brain steroid profiles, which may account for the better outcome of pseudopregnant females, compared with males after TBI. Pseudopregnant females have high levels of PROG and its 5α-reduced metabolites, which have been demonstrated to be neuroprotective. Males have low levels of PROG, its precursor, and its reduced metabolites and significant levels of testosterone, its precursor, and its metabolites.

Effect of TBI on steroid levels in male and pseudopregnant females

In addition to differences in the steroid profiles in the brain at the time of injury, the subsequent effects of TBI on plasma and brain steroid levels in males and pseudopregnant females are different. Six hours after TBI, the injury induced an increase of 5α-DHPROG and 3β,5α-THPROG in brain of pseudopregnant females and PREG, PROG, and 5α-DHPROG in brain of male rats. The increases of PREG and PROG levels in plasma of males were not statistically significant. In males, injury led to an increase in both the synthesis and metabolism of PROG. It is possible that an activation of the hypothalamo-pituitary-adrenal axis by the stress caused by TBI led to the stimulation of PREG, PROG, and corticosterone secretion in the blood and to a subsequent accumulation in the brain. The increase of PREG and PROG in male brain may also be due to a local brain increase in steroidogenic enzyme activities and/or an up-regulation of the proteins involved in the intramitochondrial transport of cholesterol, the rate-limiting step in steroidogenesis, such as steroidogenic acute regulatory protein and peripheral benzodiazepine receptor. Indeed, peripheral benzodiazepine receptor and steroidogenic acute regulatory protein have been shown to be up-regulated in different models of nervous system injuries including TBI (45–49).

The rate of increase of 5α-DHPROG in the injured brain was more substantial in males (+189% in the lesion site, +145% distal to the lesion site) than pseudopregnant females (+59% in the lesion site). Because 5α-DHPROG and its metabolite could have neuroprotective effects after TBI (7, 8), the stimulation of 5α-DHPROG production may be more necessary in males than pseudopregnant females because the absolute values of brain 5α-DHPROG levels are 100 times higher in pseudopregnant females than males. The observed increase of 5α-DHPROG levels in the brain of both males and pseudopregnant females after TBI is very likely due to an increase in its local synthesis because the increase in brain

levels is not correlated with an increase in plasma levels, and the observed increase was not homogeneous for all brain regions. Twenty-four hours after TBI, most steroid levels in brain and plasma were similar to those in sham-operated animals, suggesting a transitory effect of TBI on steroid levels. A previous report showed an increase in PROG, 5α-DHPROG, and 3α,5α-THPROG in male rat cerebral cortex after lateral fluid percussion (50). The increase was observed in the perifocal but not focal site. However, in this study there was no information concerning plasma steroid levels, and the measurements were done after infusion of large PREG-sulfate amounts, thus providing no information concerning changes in endogenous steroids.

Recently we have shown in male rats an increase in PROG, 5α-DHPROG, and 3α,5α-THPROG levels after spinal cord injury (16). Thus, the increase of PROG and its reduced metabolites in the male rat nervous system may be a general mechanism that could play a pivotal role in the capacity of the nervous system to respond to the consequences of injury. The 5α-reduction of PROG yielding to 5α-DHPROG then to 3α,5α-THPROG seems to be a major metabolic pathway of PROG in the nervous system. The 5α-reduction is the highest rate-limiting step in this pathway (51, 52). This pathway is activated after TBI (our present study) and spinal cord injury (16). These observations suggest that the local reduction of PROG in brain and spinal cord may be of particular relevance after injury and raise the question of the role of the metabolite reduction of PROG in the injured nervous system. Like our previous results for the spinal cord (16), the present results showed a high correlation between the levels of the product 5α-DHPROG and its precursor PROG. Indeed, in male rats, when levels of PROG were low, the levels of 5α-DHPROG measured in spinal cord and brain were low. When PROG levels were high, after treatment of males with PROG or after induction of pseudopregnancy, there was a high production of 5α-DHPROG and a subsequent 3β,5α-THPROG formation. The type 1 isoenzyme of 5α-reductase has apparent Michaelis constant (Km) values for steroid substrate in the micromolar range, whereas the Km of type 2 is in the nanomolar range. If we take into account the levels of PROG present in brain and spinal cord and the Km of these isoenzymes (53), it can be speculated that, in male rats, the 5α-reductase type 2 is the most reactive enzyme, whereas both isoenzymes 1 and 2 of 5α-reductase may contribute to the 5α-DHPROG synthesis after treatment with PROG and in pseudopregnant females.

The effects of PROG and its reduced metabolites can be mediated by the intracellular receptors, progesterone receptor (PR; PROG and 5α-DHPROG) or membrane receptors [25-Dx (54); PR (55, 56)], or interaction with γ-aminobutyric $acid_A$ receptors via 3α,5α-THPROG (57). 3β,5α-THPROG may antagonize the effects of 3α,5α-THPROG (58–60). The overall effects in the nervous system may be dependent on the available amounts of the steroids on the expression pattern of the different receptors and the binding affinity of each receptor system. After injury, 5α-DHPROG levels increased in spinal cord and brain of both males and females. This increase may be of high physiological importance because 5α-DHPROG can activate the PR (61) and it is the precursor of 3α,5α-THPROG and its isomer 3β,5α-THPROG. 3α,5α-THPROG has been shown to be neuroprotective after TBI (62), promote neuronal survival (63, 64), and regulate the proliferation of neural progenitors (65, 66). 5α-pregnane steroids interact with γ-aminobutyric $acid_A$ receptors (67, 68) but can also interact with the PR (61, 69). The increase in the rate of conversion of PROG to its reduced metabolites in the injured nervous system may increase the cross-talk mechanism between membrane and genomic PROG effects.

In male rats, in addition to stimulating PROG synthesis and metabolism, the injury induced an important transient decrease of testosterone in brain and plasma 6 h after TBI. This decrease could be due to a disruption of the hypothalamo-pituitary-gonadal axis. Indeed, in men, endocrine deficits are rather common after TBI, and isolated hypotestosteronemia is frequent. Analysis of posttraumatic endocrine deficits in a series of severe TBIs showed an incidence of 28% of hypotestosteronemia. All were of central origin (low testosterone and low or normal LH) due to hypothalamic or pituitary origin (70). Hypothalamic hypogonadism after head injury has been reported by other authors (71–73). A decrease in serum levels of testosterone was also reported in spinal cord-injured men (74). In addition to the disruption of the hypothalamo-pituitary-gonadal axis, the observed decrease of testosterone in brain and plasma could also be due in part to the increased levels of 5α-DHPROG. Indeed, 5α-DHPROG, which is not involved in the biosynthesis pathway of testosterone, may be a substrate for P450c17 enzyme (75, 76) as well as PREG and PROG, which are precursors of testosterone (Fig. 1). Thus, the increased levels of 5α-DHPROG after TBI may lead to an inhibitory competition for P450c17 binding, causing a decrease in testosterone synthesis. This hypothesis is supported by the decreased plasma levels of Δ^4-androstenedione, the direct precursor of testosterone. Finally, the observed decrease in testosterone may be a consequence of the stress caused by TBI. Indeed, several studies have shown that stress impaired steroidogenesis and induced a reduction of testicular and circulating testosterone by affecting the activity of P450c17 (77–79).

In conclusion, we have shown a substantial difference in levels of PROG and its reduced metabolites in brain and plasma of male and pseudopregnant female rats, which could account for the better outcome from TBI in pseudopregnant females, compared with males. Six hours after TBI, the brain levels of 5α-DHPROG increased in both males and pseudopregnant females, suggesting that the local reduction of PROG in brain may be of particular relevance after TBI and raising the question of its role in the injured nervous system.

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