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Injury Elicited Increase in Spinal Cord Neurosteroid Content Analyzed by Gas Chromatography Mass Spectrometry

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The effects of spinal cord injury (SCI), combined with castration and adrenalectomy, and of progesterone (PROG) treatment on neurosteroid levels and steroidogenic enzyme expression were investigated in the adult male rat spinal cord (SC). Steroid levels were quantified by gas chromatography/mass spectrometry in SC and plasma, and mRNAs of enzymes by quantitative real-time RT-PCR. The levels of pregnenolone (PREG), PROG, 5α-dihydroprogesterone, 3α,5α-tetrahydroprogesterone increased in SC 75 h after transection without significant increase in the plasma. After combined adrenalectomy and gonadectomy, significant levels of PREG and PROG remained in the SC, suggesting their local biosynthesis. In the SC of adrenalectomized and gonadectomized rats, there was an increase of PREG 24 h after SCI, followed at 75 h by a concomitant increase in its direct metabolite, PROG. These observations are consistent with a sequential increase of PREG biosynthesis and its conversion to PROG within the SC in response to injury. However, no significant change in P450-side chain cleavage and 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase mRNA levels was observed after SCI. Systemic PROG treatment after SCI, resulted in a very large increase in PROG, 5α-dihydroprogesterone, and 3α,5α-tetrahydroprogesterone in both plasma and SC. Furthermore, high levels of 3β,5α-tetrahydroprogesterone were detected in SC, whereas their plasma levels remained barely detectable. Because the ratio of reduced metabolites to PROG was 65-times higher in SC than in the plasma, it appears likely that reduced metabolites mainly originated from local biosynthesis. Our results strongly suggest an important role for locally biosynthesized neurosteroids in the response of the SC to injury. (*Endocrinology* 147: 1847–1859, 2006)

BOTH THE STEROIDOGENIC endocrine glands (gonads and adrenal glands) and the local biosynthesis contribute to the pool of steroids present in the nervous system. Therefore, changes in plasma levels of steroids do not necessarily reflect changes in nervous tissue levels. Pregnenolone (PREG) and progesterone (PROG) can be synthesized in the nervous system and, thus, they have been termed "neurosteroids" (1). There are two requirements for a steroid to qualify as a neurosteroid: 1) persistence of the steroid in nervous tissue in the absence of steroidogenic endocrine glands and 2) expression and activity of the enzymes involved in its biosynthesis in the nervous system. The steroidogenic acute regulatory protein (StAR) and the peripheral-type benzodiazepine receptor (PBR) play a key role in the regulation of the cholesterol transport from the outer to the inner mitochondrial membrane, where the first step of steroid biosynthesis takes place. PBR is a mitochondrial protein that might function as a channel for cholesterol. PBR regulates the mitochondrial intermembrane transport of cholesterol through interactions with StAR (2, 3), thereby regulating the biosynthesis of PREG. Different studies have shown that the nervous system has the capacity to biosynthesize and metabolize PROG (4, 5). The biosynthesis of PROG from cholesterol involves two enzymatic steps: the conversion of cholesterol to PREG by cytochrome P450 side chain cleavage (P450scc) and the conversion of PREG to PROG by 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD). PROG is further metabolized to 5α-dihydroprogesterone (5α-DHPROG) by the steroid 5α-reductases (two distinct isozymes 1 and 2) and to 3α,5α-tetrahydroprogesterone (3α,5α-THPROG, allopregnanolone) by the 3α-HSD. In addition, 5α-DHPROG can be reduced to 3β,5α-tetrahydroprogesterone (3β,5α-THPROG, isoallopregnanolone) by the 3β-hydroxysteroid oxidoreductase (Fig. 1).

In the spinal cord (SC), significant levels of PREG and PROG remain present after castration and adrenalectomy (6). Expression, cellular distribution, and bioactivity of the key steroidogenic enzyme, cytochrome P450scc have been determined in the SC (7, 8). We have recently shown a wide

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Abbreviations: ADX, Adrenalectomized/adrenalectomy; Ct, threshold cycle; CTL, control sham-operated; 5α-DHPROG, 5α-dihydroprogesterone; GDX, gonadectomized; GS/MS, gas chromatography/mass spectrometry; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase; 3α-HSOR, 3α-hydroxysteroid oxidoreductase; P450scc, cytochrome P450 side chain cleavage; PBR, peripheral-type benzodiazepine receptor; PREG, pregnenolone; PROG, progesterone; SC, spinal cord; SCI, SC injury; SIM, single ion monitoring; StAR, steroidogenic acute regulatory protein; 3α,5α-THPROG, 3α,5α-tetrahydroprogesterone; 3β,5α-THPROG, 3β,5α-tetrahydroprogesterone; TRX, transection at thoracic level T10.

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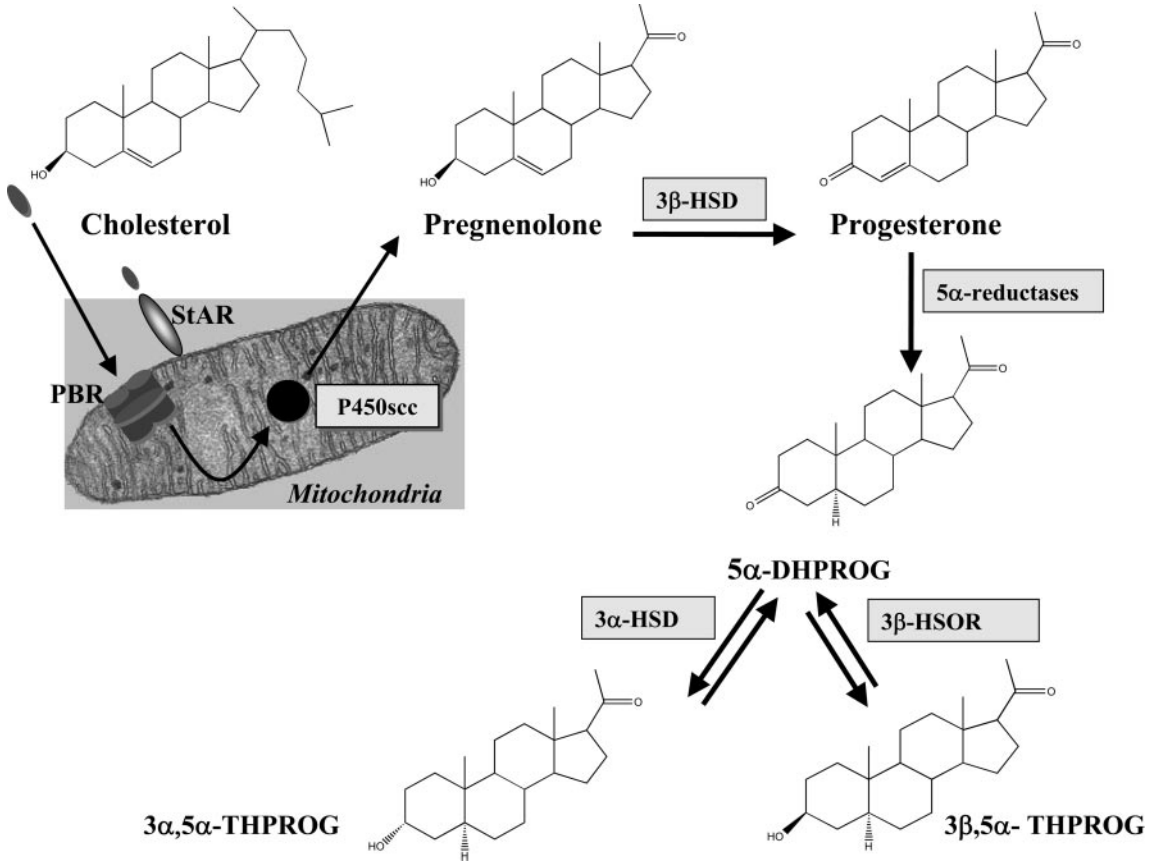

FIG. 1. Pathways of PROG synthesis and metabolism to its 5α-reduced derivatives. 3β-HSOR, 3β-Hydroxysteroid oxidoreductase.

distribution of 3β-HSD enzyme in SC and its expression in both motoneurons and small neurons of the dorsal horn (6). The expression and anatomical and cellular localization of 5α-reductase 1, 5α-reductase 2, and 3α-hydroxysteroid oxidoreductase (3α-HSOR) enzymes have been more recently demonstrated in the SC (9, 10). 5α-reductase 1 was detected in glial cells, whereas 5α-reductase 2 and 3α-HSD immunoreactivities were found in glia and neurons (10).

These different studies demonstrated that SC possesses the enzymatic set to synthesize PROG and its potent neuroactive metabolites that may control spinal processes.

PREG, PROG, and its 5α-reduced derivatives are neurosteroids and also neuroactive molecules that exert pleiotropic effects throughout the nervous system. PREG and PROG are neuroprotective: they reduce damage resulting from primary and secondary SC injury (SCI) and promote neurological recovery (11, 12). Improvement in locomotor activity after PREG or PROG treatments was paralleled by reduction in histopathological changes. In a previous work, we have shown that PROG regulates some key features of neuronal and glial functions after SCI (13–16). It is likely that multiple mechanisms are involved in the neuroprotective and trophic effects of PROG: it can be effective *per se* after binding to specific nuclear PROG receptors or membrane receptors (25-Dx or mPR) or it can be reduced to 5α-DHPROG, which also binds with high affinity to intracellular PROG receptors. 5α-DHPROG can be reduced to 3α,5α-THPROG, which is a potent positive modulator of $GABA_A$ receptors (17, 18). 5α-DHPROG can also be converted to 3β,5α-THPROG, which is inactive on $GABA_A$ receptors, but can antagonize the effect of 3α,5α-THPROG (19).

Because PREG, PROG, 5α-DHPROG, 3α,5α-THPROG, and 3β,5α-THPROG are metabolically associated, changes of the endogenous levels of a specific neurosteroid may reflect *in vivo* regulation of its biosynthesis and metabolism, which in turn may provide insights into its physiological roles. In this study, we have investigated the steroid levels and their regulation by gas chromatography/mass spectrometry (GC/MS) and steroid enzyme expression by quantitative real-time RT-PCR in rat SC. The aim was to determine: 1) levels of PREG, PROG, and its metabolites in the different segments of the SC and to compare them to plasma levels; 2) the effect of SCI on these steroid levels 75 h after transection; and 3) whether the observed increase in steroid levels after SCI is due to local synthesis. For this purpose, we have studied 1) the effect of SC transection on PREG and PROG levels in castrated adrenalectomized rats at 24 and 75 h after transection; 2) the effect of SC transection on P450scc and 3β-HSD mRNAs expression in the SC after 24 and 75 h; and 3) the levels of the parent steroid PROG and its reduced metabolites in plasma and SC after systemic treatment with PROG.

Materials and Methods

Animals and surgical procedures

Adult Sprague Dawley male rats (200–250 g) were randomly divided in five different groups: 1) control sham-operated rats (CTL); 2) rats with total SC transection at thoracic level T10 (TRX); adrenalectomized

(ADX)/gonadectomized (GDX) male rats either 3) sham operated (ADX/GDX) or 4) with SC transection (ADX/GDX/TRX); and 5) rats with SC transection and treated with PROG (4 mg/kg·d for 3 d) (TRX + PROG).

For SC transection (TRX), animals were anesthetized with an ip injection of 40 mg/kg of ketamine (Imalgène, Rhone Mérieux, France) and 1.6 mg/kg of acepromazine (Vetranquil, Sanofi, France), and they were divided into sham and SC-transected groups. In the last case, after careful laminectomy, a complete SC transection was carried out at thoracic level T10 using the sharp edge of a 25G needle (20). Sham-operated controls were laminectomized, but the SC was left intact. Four experiments were designed (Fig. 2).

Experiment 1 was designed to measure by GC/MS the basal levels of steroids in SC and plasma (set 1 of animals) and to check the expression of the mRNAs of steroidogenic enzymes (set 2) in control animals.

Experiment 2 was designed to study the effect of transection on steroid levels and steroidogenic enzyme expression in the SC in intact adult male Sprague Dawley rats. Two sets of animals were prepared: set 1 for the measurements by GC/MS of the steroid levels at 75 h after injury, and set 2 for RNA preparation to study the profile of the expression of steroidogenic enzymes in the SC 24 and 75 h after injury by using quantitative real-time PCR.

Experiment 3 was designed to check whether the increase in steroid levels after SC transection (observed in experiment 2) persisted in the absence of the steroidogenic endocrine glands. Adult male Sprague Dawley rats were ADX 1 wk after castration, and transection of the SC was performed 1 wk after ADX. The animals were killed by decapitation 24 or 75 h after SC transection.

Experiment 4 was designed to measure the levels of steroids in transected SC after PROG treatment. Injured male rats received four injections of 4 mg/kg PROG at times 1 h (ip) and sc at 24, 48, and 72 h after lesion. This paradigm of PROG treatment is the one that has previously been shown to prevent neuronal loss after brain injury and SCI (12, 21), and to modulate motoneurons and glial cell markers after SCI (13, 14, 15, 22).

For experiments 1, 2, and 3, four segments of SC (cervical, thoracic, lumbar, and sacral) were dissected out and used for the steroid analysis

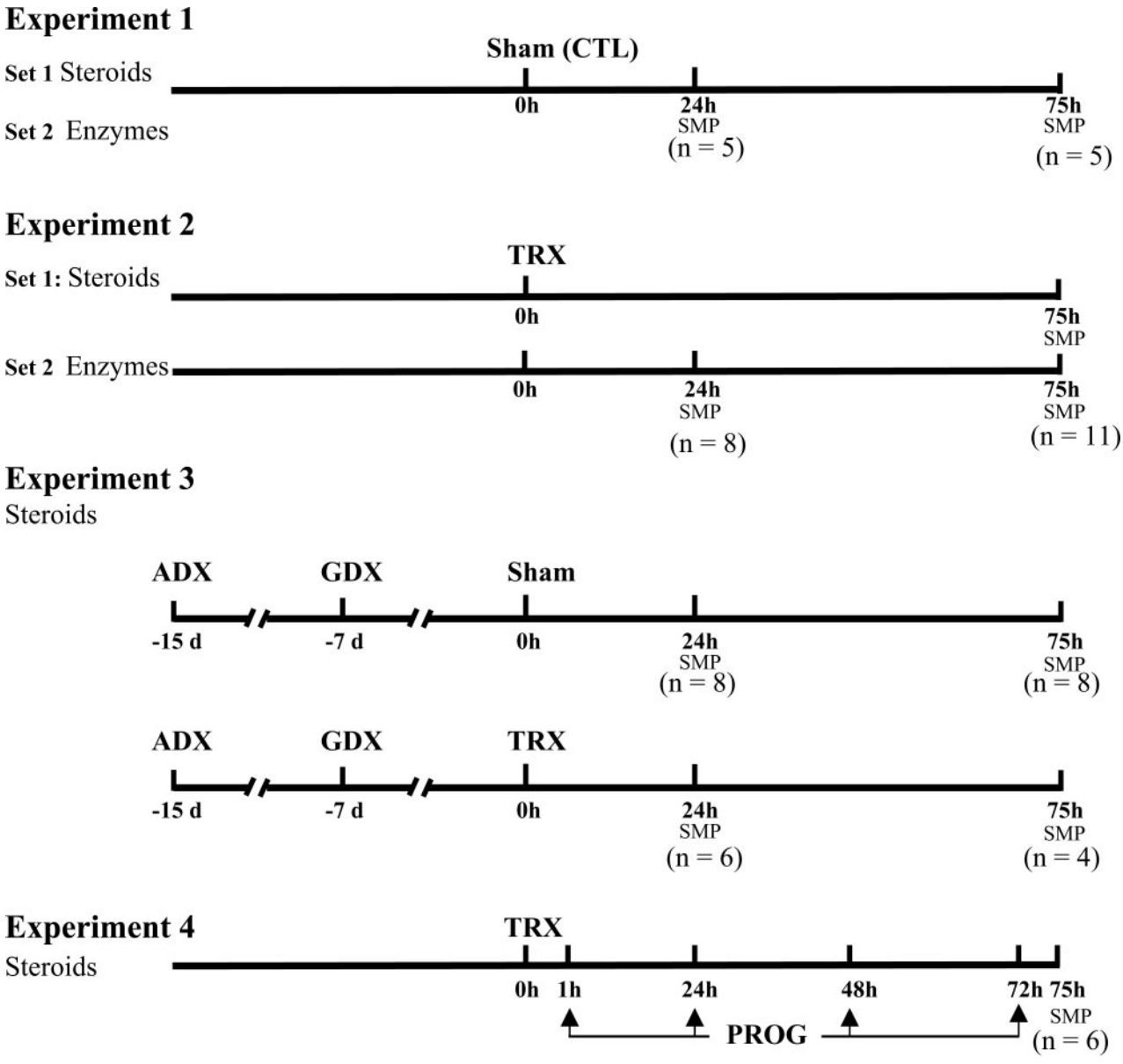

FIG. 2. Summary of experimental groups and time course of the different surgeries and treatments. (n), number of animals per group; PROG, 4 mg/kg; SMP, sampling.

by GC/MS. The analysis of the expression of the steroidogenic enzymes mRNA (sets 2 of experiments 1 and 2) and the steroid levels after PROG treatment (experiment 4) have been limited to the lumbar segment. This level was chosen because lesions destroying descending tracts lead to transynaptic degeneration of motoneurons below the lesion site (23). Additionally, several genes have already been shown to be regulated in glial cells and motoneurons in the lumbar region after transection and PROG treatment after transection at T10 (13, 14, 22).

Animal care. In spinally injured rats, bladder expression was assisted by massage at least twice a day. Animals were carefully monitored for evidence of urinary tract infection or any other sign of disease. Daily inspection included examination of the laminectomy site for evidence of infection. Animal experimentation was conducted in accordance with the accepted standards of human animal care as outlined in the ethical guidelines, and it was approved by the Institute's Animal Care and Use Committee.

P450scc, 3β-HSD, 5α-reductase type 1 and 2 and 3α-HSOR mRNA expression: analysis by RT-PCR and real-time quantitative RT-PCR

Animals were prepared as described above for experiment 1, set 2 and experiment 2, set 2. Lumbar regions of SC were sampled, frozen on dry ice, and stored at −80 C until use. RNA was extracted from individual segments of SC.

RNA extraction. Frozen tissues were grinded into powder in a mortar precooled with liquid nitrogen. Total RNA was then extracted using TRIzol reagent (Life Technologies, Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions. The concentration and purity of total RNA was determined by measuring the optical density at 260 and 280 nm. All samples were precipitated with ethanol, then dissolved in distilled water at a concentration of 1 μg/μl, and their quality was verified by gel electrophoresis.

RT. Total RNA was subjected to Dnase 1 (Stratagene, La Jolla, CA) treatment (10 U for 15 min at 37 C) to remove residual contaminating genomic DNA. cDNA templates for PCR amplification were synthesized from 2 μg of total RNA using a SuperScript II Rnase H reverse transcriptase kit (Life Technologies, Inc./BRL, Cergy Pontoise, France) for 90 min at 42 C in the presence of random hexamer primers.

Primers. Primers for RT-PCR amplification and quantitative real-time RT-PCR were designed using Oligo Primer Analysis Software version 6.54 (Molecular Biology Insights Inc., Cascade, CO). Their sequences and final concentration used for amplification are indicated in Table 1.

RT-PCR amplification. RT-PCR amplification was used to check the expression of the mRNAs of P450scc, 3β-HSD, 5α-reductase type 1 and 2, and 3α-HSOR enzymes. Each PCR contained 200 ng cDNA template, 1× *Taq* DNA polymerase buffer, 200 μM of each deoxynucleotide triphosphate, 1 U *Taq* DNA polymerase (ATGC), and the appropriate concentrations of specific primers as indicated in Table 1, in a total volume of 50 μl. The conditions of amplification were: 2 min at 94 C, followed by 35 cycles at 94 C for 1 min, 56 C for 1 min, and 72 C for 1 min. Aliquots of the amplified products and 100-bp DNA ladder were separated on a 1.2% agarose gel and visualized by ethidium bromide staining. Amplified fragments were extracted from the gel using the JETSORB Kit. The purified fragments were resuspended in water, and their sequence determined (Biofidal, Vaulx en Velin, France).

Real-time quantitative PCR (RT-qPCR). Neurosteroidogenic enzymes P450scc and 3β-HSD mRNAs and the housekeeping gene 26S rRNA were quantified using absolute QPCR SYBER Green ROX Mix (Abgene, Courtaboeuf, France) following the instructions of the manufacturer. 26S rRNA was chosen as housekeeping gene based on the similarity of expression across all sample templates. Quantitation of relative gene expressions was performed by the method of RT-qPCR using the ABI PRISM 7000 sequence Detection System (Applied Biosystems, Foster City, CA), which combines PCR, cycle-fluorescence detection, and analysis software for high throughput quantification of nucleic acid sequences. Reactions are characterized by the cycle number at which amplification of a PCR product is first detected. The higher the copy number of the nucleic acid target, the sooner a significant increase in SYBR I green fluorescence is observed. Relative gene expression data were calculated using the comparative threshold cycle (Ct) method (24). Briefly, the Ct was determined for each gene of interest. For each sample, ΔCt was calculated as the difference in Ct between target mRNA and 26S rRNA (housekeeping gene). ΔΔCt was calculated as the difference between ΔCt for each sample and the average ΔCt for the control group (baseline). The change in target mRNA, relative to baseline, was calculated as $2^{-(\Delta\Delta Ct)}$. Changes in mRNA expression were expressed as percent of the control group (CTL).

Validation of RT-qPCR. Specificity of PCR amplification was confirmed by melting curve analysis. In addition, PCR products were controlled with high-resolution gel electrophoresis and subsequent sequencing. All PCR amplifications lead to a single and specific product. Linearity and efficiency of PCR quantification were validated before quantification (Table 2). The accuracy of mRNA quantification depends on the linearity and efficiency of PCR amplification. Both parameters were assessed using standard curves generated by increasing amounts of cDNA. Relationship between the threshold cycle (C_T) and the logarithm of the cDNA concentration were studied according to 1) the correlation coefficient and 2) the amplification efficiency. Correlation coefficients (r) confirm the linear relationship between the threshold cycle (C_T) and the logarithm of the cDNA concentration. Standard curves, using five points, diluted over a 100-fold range, always led to a high linearity with all primer sets (Table 2). Relative gene expression was calculated by the ΔC_T method (24). This method is based on similar amplification efficiency rates between target and reference genes and consider PCR efficiency close to 1 when PCR have been properly optimized. The PCR efficiency (*Ex*) was calculated using the equation $Ex = (10^{-1/\text{slope}}) - 1$ (25). The linearity and efficiency of amplification of PCR assays (Table 2) among different templates allowed an accurate quantification of P450scc and 3β-HSD mRNAs. Samples were run in duplicate, and linearity and efficiency were calculated from two independent assays.

For each amplification, 100 ng cDNA were used and PCR was performed in optimized conditions: 95 C for 15 min followed by 35 cycles at 95 C for 15 sec and 56 C for 1 min. The analysis of reaction was performed by ABI 7000 SDS software.

TABLE 1. Nucleotide sequences and concentrations of sense and antisense primers used in PCR analysis

mRNA detected	Primer orientation	Sequence 5′→3′	Real-time RT-PCR (nM)	RT-PCR (nM)
P450scc	Sense	GCTTTGGCTGGGGTGTT	25	400
	Antisense	GCTTGAGAGGCTGGAAGTTG		
3β-HSD	Sense	TGGGAGCATCCTGAAAAAT	200	400
	Antisense	TTGTGACTTGGGGTCTC		
5α-Red 1	Sense	CGACCTGCCTGGTTCATACA		400
	Antisense	GGTCACCCAGTCTTCAGCAT		
5α-Red 2	Sense	GTCCTGCTGGCTCTCTTCTC		400
	Antisense	CAGGCTTCCTGAGCTGGCG		
3α-HSD	Sense	CTGTGCCTGAGAAGGTTGCT		1000
	Antisense	CATGTGTCACAGATATCCAC		
26S	Sense	AGGAGAAACGGTCGTGCCAAA	200	
	Antisense	GCGCCAGCAGGTCTGAATCGT		

TABLE 2. Validation of quantitative real-time RT-PCR

	P450scc	3β-HSD	RNA 26 S
r	0.995 ± 0.005	0.993 ± 0.003	0.980 ± 0.006
Ex	0.988 ± 0.009	1.082 ± 0.180	1.060 ± 0.167

Correlation coefficients (r) confirm the linear relationship between the threshold cycle and the logarithm of the cDNA concentration. The PCR efficiency (Ex) was calculated using the equation $Ex = (10^{-1/slope}) - 1$ (25). Similar amplification efficiency rates between target and reference genes were obtained (Ex close to 1). The r and Ex values showed that PCR conditions have been properly optimized.

Measurement of steroid levels by gas chromatography coupled to mass spectrometry

PREG, PROG, 5α-DHPROG, 3α,5α-THPROG, and 3β,5α-THPROG levels were determined by GC/MS according to the protocol described by Liere *et al.* (26) with minor modifications. Briefly, steroids were extracted from individual SC regions (the weight range was 60–250 mg of tissue) and plasmas (1 ml) by adding 10 volumes of methanol. The internal standards epietiocholanolone (for PREG, 3α,5α-THPROG, and 3β,5α-THPROG), 19-nor PROG (for PROG), and $^2H_6$-5α-DHP (for 5α-DHPROG) were introduced into the extract for steroids quantification. Corticosterone measurements were performed only in adrenal glands, and trideuterated cortisol was added as the internal standard. Samples were purified and fractionated by solid-phase extraction on C18 columns (International Sorbent Technology, Mid Glamorgan, UK) with a recycling procedure (27). This step permits to isolate unconjugated steroids from their conjugated counterparts (sulfates, glucuronides, fatty acid esters). The unconjugated steroids, containing solid-phase extraction fraction, were filtered and submitted to HPLC in straight phase coupled to a 202 model Gilson fraction collector (24). 5α-DHPROG was collected in a time scale of 3–10 min and PREG, PROG, 3α,5α-THPROG, and 3β,5α-THPROG in a time scale 10–31 min. Corticosterone was collected in a third HPLC fraction from 31–45 min. All of these groups of steroids need to be separated by HPLC before their chemical structure-specific derivatization. The 5α-DHPROG-containing fraction was submitted to a mixture MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide)/NH_4I/DTE (1000:2:5 vol/vol/vol) to form the 3, 20-ditrimethylsilyl ether dienol of 5α-DHPROG. The second fraction of HPLC containing PREG, PROG, 3α,5α-THP, and 3β,5α-THP was acylated with heptafluorobutyric anhydride as derivatization reagent as described by Liere *et al.* (26). The third fraction of HPLC containing corticosterone was derivatized with heptafluorobutyric anhydride to synthesize the 3-dienol, 21-diheptafluorobutyrates of corticosterone with a dehydratation at C11 and formation of a double bond between C9 and C11 and the 3-dienol, 21-diheptafluorobutyrates of cortisol with double bonds at C9–C11 and C13–C14 and a 17β-methyl 18-nor-substructure.

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 ramped between 50–330 C. 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. The mass spectra of the investigated steroids are shown in Fig. 3. Quantification was performed in single ion monitoring (SIM) mode according to the major diagnostic ion, as indicated in Table 3, and to the retention time of each derivatized steroid.

Before measuring the steroids in the samples, validation of the analytical protocol (slightly modified since our previous article; Ref. 24) has first been done on a pool of adult male rat central nervous tissue and plasmas. Several aliquots of extracts from brain (10, 20, 50, 100, 200, and 300 mg of tissue) and plasma (corresponding to 50, 100, 200, 500, and 1000 μl) were processed with this protocol (n = 6 for each measure). The minimal weight of brain tissue necessary for steroid detection was 20 mg for PREG and PROG, 300 mg for 5α-DHPROG, and 3α5α-THPROG, whereas 3β5α-THPROG could not be detected. In plasma, the minimal values were 100 μl for PREG and PROG, whereas 5α-DHPROG, 3α5α-THPROG, and 3β5α-THPROG could not be not detected from 1 ml of plasma. Thus, the correlation coefficients could only be determined for PREG and PROG in all the tested nervous tissue weight range and in all the plasma volume range. The major results of this validation (basal values, coefficient of variation, coefficient of correlation) are summarized in Table 4.

According to the GC/MS detection limit, dilution factor and recovery, the lowest PREG, PROG, 5α-DHPROG, 3α,5α-THPROG, and 3β,5α-THPROG concentrations that can be measured by GC/MS on 150 mg of SC are 0.13, 0.066, 0.33, 0.33, and 0.33 ng/g and 0.02, 0.01, 0.05, 0.05, and 0.05 ng/ml in 1 ml of plasma, respectively.

Statistical analysis

All data were analyzed by a commercially available program (Prism 3.0; GraphPad, San Diego, CA). Data were processed using one-way ANOVA, followed by *post hoc* comparisons with the Newman-Keuls test, two-way ANOVA followed by Bonferroni posttest, or Student's *t* test depending of the groups to be compared (see details in figure legends). Statistical significance was noted when the probability of type I error was less than 0.05.

Results

Steroidogenic enzymes involved in PROG synthesis and metabolism are expressed in SC of adult male rats

Results from experiment 1, set 2 (see *Materials and Methods* and Fig. 2) showed that the complete neurosteroidogenic enzymatic system necessary for the synthesis of 3α,5α-THPROG from cholesterol (Fig. 1) is expressed in SC. Figure 4 shows the expression of the mRNAs of P450scc, the enzyme involved in the conversion of cholesterol into PREG; 3β-HSD, the enzyme involved in the conversion of PREG to PROG; 5α-reductase type 1 and 5α-reductase type 2, the enzymes involved in the reduction of PROG to give arise to 5α-DHPROG; and 3α-HSD, the enzyme involved in the conversion of 5α-DHPROG into 3α,5α-THPROG in positive control tissues (adrenals, testis, or liver) and in the lumbar region of SC as analyzed by RT-PCR.

Sequencing of the amplified products and analysis by the NCBI Blast program showed that the nucleotide sequences of the amplified fragments were 100% homologous to the expected fragments in the sequences of P450scc (GI:203638), 5α-reductase type 1 (GI:1336834), 5α-reductase type 2 (GI:49225970), and 3α-HSD (GI:19924086). The primers used for 3β-HSD were designed to amplify the four rat 3β-HSD isoforms. Sequencing of the amplified fragment showed that the isoform expressed in SC was 100% homologous to 3β-HSD type 1 (GI:531215).

PREG, PROG, 5α-DHPROG, and 3α,5α-THPROG levels in SC and plasma of adult male rats

The basal concentrations of steroids were measured in different segments of SC (cervical, thoracic, lumbar, and sacral regions) and plasma by GC/MS (experiment 1, set 1; see *Materials and Methods* and Fig. 2). Steroids were identified according to characteristic ions (Fig. 3 and Table 3) in conjunction with their GC retention time, and their levels were determined using calibration curves. As shown in Fig. 5, significant levels of PREG, PROG, 5α-DHPROG, and 3α,5α-THPROG, were detected in the SC and plasma of the intact male rats.

The level of PREG was much higher in the SC than in plasma. The levels were higher in the cervical, thoracic, and

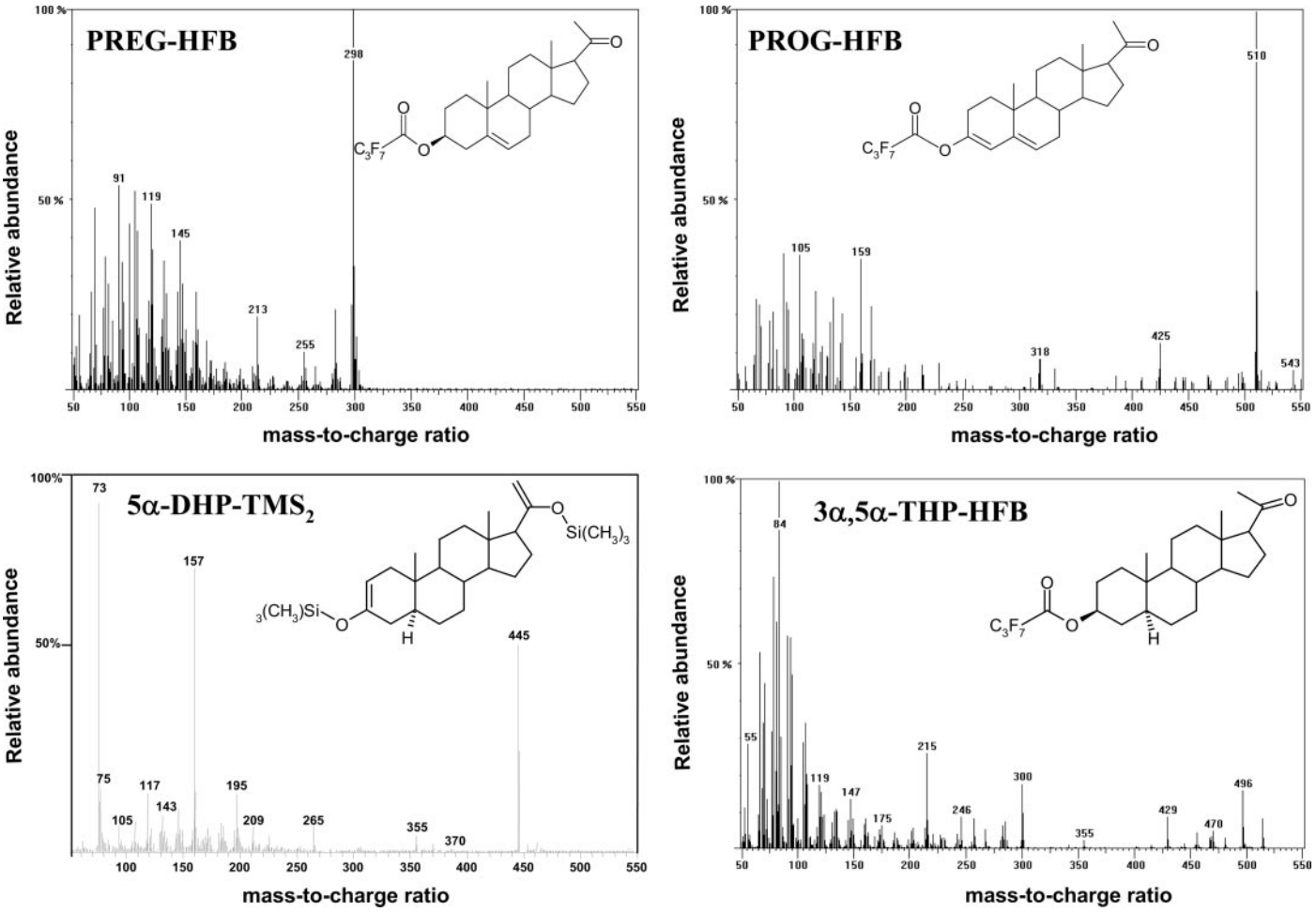

FIG. 3. Mass spectra of PREG-HFB, PROG-HFB, 5α-DHPROG-TMS$_2$, and 3α,5α-THPROG-HFB (identical to that of 3β,5α-THPROG-HFB). PREG, PROG, 3α,5α-THPROG and 3β,5α-THPROG were derivatized with heptafluorobutyric anhydride (HFB) to form PREG-HFB, PROG-HFB, 3α,5α-THPROG-HFB and 3β,5α-THPROG-HFB, respectively, whereas 5α-DHPROG was derivatized with a mixture of MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide)/NH4I/DTE (1000:2:5 vol/vol/vol) to form the 3, 20 ditrimethylsilyl ether dienol of 5α-DHPROG (5α-DHPROG-TMS2). Ionization was performed by electronic impact with ionization energy of 70 eV. The temperature of the ionization chamber was fixed at 180 C. SC and plasma samples were analyzed in SIM mode on the diagnostic ions of each steroid of interest (see Table 3).

sacral segments than in the lumbar segment ($P < 0.05$) (Fig. 5B, PREG, *white bars*). Similar levels of PROG were detected in plasma and the cervical, thoracic, and sacral segments of the SC. These levels were higher than that detected in the lumbar region ($P < 0.05$) (Fig. 5B, PROG, *white bars*). Levels of 5α-DHPROG were higher in the lumbar region of the SC than in plasma. The levels of 5α-DHPROG in the cervical, thoracic, and sacral regions were below the threshold of detection of our technical procedure (*i.e.* 0.5 ng/g), whereas significant levels were measured in the lumbar region (Fig. 5B, 5α-DHPROG, *white bars*). This observation may reflect an active conversion of PREG and PROG in the lumbar region as relatively low levels of PREG and PROG were detected in this region. Indeed, 5α-DHPROG is a direct metabolite of PROG, itself a direct metabolite of PREG. The levels of 3α,5α-THPROG were higher in the cervical, thoracic, and sacral regions of the SC than in plasma. The levels in the lumbar region were low comparatively to the other regions of the SC

TABLE 3. Analysis of steroids by electronic impact GC/MS

Steroid	Molecular mass (Da)	Derivatized steroid	Molecular mass of derivatized steroids (Da)	Diagnostic ions (m/z)
PREG	316	PREG-HFB	512	283 and **298**
PROG	314	PROG-HFB	510	495 and **510**
3α,5α-THPROG	318	3α5α-THP-HFB	514	496 and **514**
3β,5α-THPROG	318	3β5α-THP-HFB	514	496 and **514**
5α-DHP	316	5α-DHP-TMS$_2$	460	**445** and 460
Corticosterone	346	Δ_{9-11} Corticosterone-HFB$_2$	720	**705** and 720

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.

TABLE 4. Validation of steroid measurements in male rat nervous tissue and plasma by GC/MS

	PREG	PROG	5α-DHPROG	3α,5α-THPROG	3β,5α-THPROG
Brain					
Basal values (ng/g ± SEM)	4.91 ± 0.27	0.36 ± 0.01	0.75 ± 0.10	0.41 ± 0.05	ND
Coefficient of variation (%)	13	4	10	21	
Coefficient of correlation	0.9967	0.9961			
Plasma					
Basal values (ng/ml ± SEM)	0.58 ± 0.03	2.17 ± 0.07	ND	ND	ND
Coefficient of variation (%)	10	7			
Coefficient of correlation	0.9981	0.9996			

Correlation coefficients (r) could only be calculated for PREG and PROG and they confirm the linear relationship between the rat brain weight (from 10 to 300 mg) or plasma volume (from 100 to 1000 μl) and the steroid amounts. In male rat brain, 5α-DHPROG and 3α,5α-THPROG could be detected from 300 mg of rat brain, whereas 3β,5α-THPROG could not be detected. In 1 ml of rat plasma, 5α-DHPROG, 3α,5α-THPROG, and 3β,5α-THPROG could not be detected. ND, Not detected (below the detection limit).

($P < 0.001$) (Fig. 5B, 3α,5α-THPROG, *white bars*). Although highest levels of 5α-DHPROG were measured in the lumbar region, only low levels of 3α,5α-THPROG could be measured. In contrast, levels of 3α,5α-THPROG were elevated in cervical, thoracic, and sacral regions, despite the fact that levels of 5α-DHPROG in these segments were below the limit of detection (compare Fig. 5B, 5α-DHPROG, *white bars* and Fig. 5B, 3α,5α-THPROG, *white bars*). This observation points to a very active conversion of 5α-DHPROG into 3α,5α-THPROG within these particular regions.

Effect of SC transection on steroid levels in adrenals, plasma, and SC of adult male rats: analysis by GC/MS 75 h after transection

Results from experiment 2, set 1 (see *Materials and Methods* and Fig. 2) showed changes in steroid levels in adrenals and SC after transection.

Adrenals (Fig. 5A). After SC transection, adrenal glands content of PREG and PROG was decreased ($P < 0.001$) with a concomitant increase of corticosterone ($P < 0.05$), whereas the small amounts of 3α,5α-THPROG remained unaltered. This finding is consistent with an activation of the pathway of corticosteroid synthesis in response to the stress induced by SC transection.

Plasma and SC (Fig. 5B). Steroid levels were increased in the SC after injury without significant changes in their plasma levels. SC content of PREG increased markedly 75 h after SCI in the four regions analyzed. Plasma levels remained very low in comparison to that measured in SC. The levels of PROG were also increased in the cervical, thoracic, and sacral regions of the SC ($P < 0.05$). Similar levels of PROG were detected in the lumbar region of the intact and injured SC. In contrast, plasma levels of PROG did not significantly change. The levels of 5α-DHPROG increased in all the SC segments. This increase was particularly significant in the lumbar region ($P < 0.0001$). The levels in the plasma remained very low. The levels of 3α,5α-THPROG increased after injury in all SC segments. No significant change in the 3α,5α-THPROG levels was detected in the plasma.

The increase of steroid levels after SCI is likely due to an increase in their local synthesis

To check whether the observed increase in steroid levels after SCI was due to an increase in their local synthesis, we studied 1) the effect of SC transection on steroid levels in the absence of the steroidogenic endocrine glands after castration and ADX (see *Materials and Methods*, experiment 3 and Fig. 2) and 2) the effects of SC transection on the expression of the key steroidogenic enzymes involved in PREG and PROG synthesis (see *Materials and Methods*, experiment 2, set 2 and Fig. 2).

After castration and ADX, significant levels of PREG, comparable to those observed in intact animals, remained present in the SC. Lower but significant levels of PROG were detected; levels of 5α-DHPROG and 3α,5α-THPROG were below the threshold of detection. Very low levels of PREG and PROG were detected in plasma. In the SC, PREG levels increased 24 h after transection, whereas there was no significant change in the PROG levels (Fig. 6). However, 75 h after transection, there was a significant decrease of PREG ($P < 0.001$) with a concomitant increase ($P < 0.01$) in its direct metabolite PROG in all regions. No significant change in PREG and PROG was detected in plasma, neither 24 nor 75 h after transection (Fig. 6).

Effect of SC transection on the levels of P450scc and 3β-HSD mRNAs local expression: analysis by RT-qPCR 24 and 75 h after transection

Large individual differences in P450scc mRNA levels were detected by RT-qPCR in SC of control animals. P450scc mRNA levels tended to increase 24 h after SC transection and to decrease after 75 h. But the differences were not statistically significant (Fig. 7A). No statistically significant change in 3β-HSD mRNA expression could be detected at 24 and 75 h after SC transection (Fig. 7B).

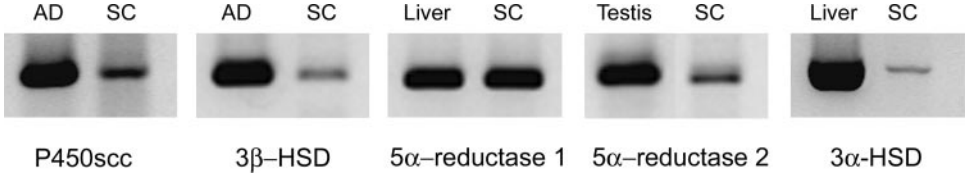

FIG. 4. Expression of P450scc, 3β-HSD, 5α-reductase isoenzymes 1 and 2, and 3α-HSD mRNAs in the lumbar region of rat SC and in positive control organs, adrenal glands, liver, and testis as analyzed by RT-PCR. AD, Adrenal gland.

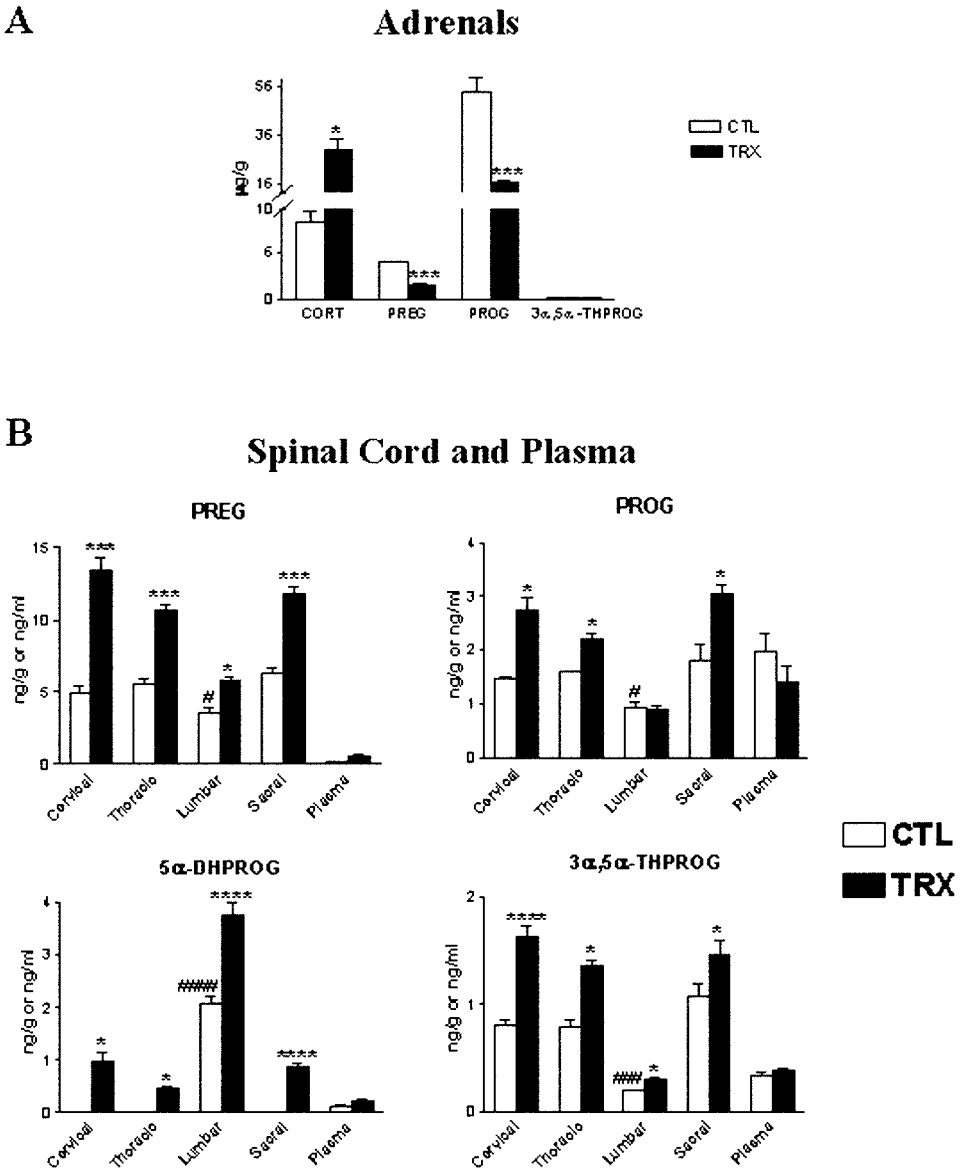

FIG. 5. Steroid levels in adrenals, SC, and plasma of intact adult male rat and after SC transection. A, Concentrations of corticosterone (CORT), PREG, PROG, and 3α,5α-THPROG in adrenals. Data represent mean of five rats for CTL and six rats for TRX. Statistical analysis: Student's *t* test. B, Concentrations of PREG, PROG, 5α-DHPROG, and 3α,5α-THPROG in cervical, thoracic, lumbar, and sacral segments of SC and plasma of intact adult male rats and after SC transection. Analysis by GC/MS. *White bars*, Basal levels. Data represent mean ± SEM of five rats. Statistical analysis: one-way ANOVA followed by Newman Keuls; #, $P < 0.05$; ###, $P < 0.001$; ####, $P < 0.0001$ *vs.* all other regions. *Black bars*, Effect of SC transection. Data represent the mean ± SEM of six rats per group. Statistical analysis: two-way ANOVA followed by Bonferroni posttest. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

Levels of steroids after SC transection and PROG treatment

The PROG treatment as applied in experiment 4 (see *Materials and Methods* and Fig. 2), resulted in a very high increase in PROG, 5α-DHPROG, and 3α,5α-THPROG levels in both plasma and lumbar region of SC. The achieved levels of PROG were higher in plasma than in the SC, whereas those of the metabolites 5α-DHPROG and 3α,5α-THPROG were higher in the SC than in the plasma. Furthermore, 3β,5α-THPROG, a metabolite of PROG that was barely detectable in the SC of control and transected rats, was present at high levels in the SC of TRX/PROG-treated rats. Very low levels of 3β,5α-THPROG were measured in the plasma (Fig. 8).

We calculated the ratio of reduced PROG metabolites (5α-DHPROG plus 3α,5α-THPROG plus 3β,5α-THPROG) to PROG in lumbar SC and in plasma, to provide an index of the conversion of the parent hormone PROG to its reduced metabolites. This ratio was very high in the SC (5.4) indicating a considerable production of reduced PROG metabolites from PROG. In the plasma, this ratio was very low (0.08). Because the ratio in SC was 65-times higher than in plasma, it is very likely that the reduced metabolites of PROG detected in SC were locally synthesized from PROG and that only a minor part is derived from the circulation.

Discussion

This study demonstrates that the SC has the capacity to synthesize PREG, PROG, 5α-DHPROG, 3α,5α-THPROG, and 3β,5α-THPROG and that the levels of these neurosteroids are modified after SCI, castration and ADX, and PROG treatment.

We have indeed measured significant levels of PREG, PROG, and 3α,5α-THPROG in different segments of SC (cervical, thoracic, lumbar, and sacral) much higher than in plasma. In addition, the complete cohort of enzymes required for the synthesis of PREG, PROG, 5α-DHPROG, and

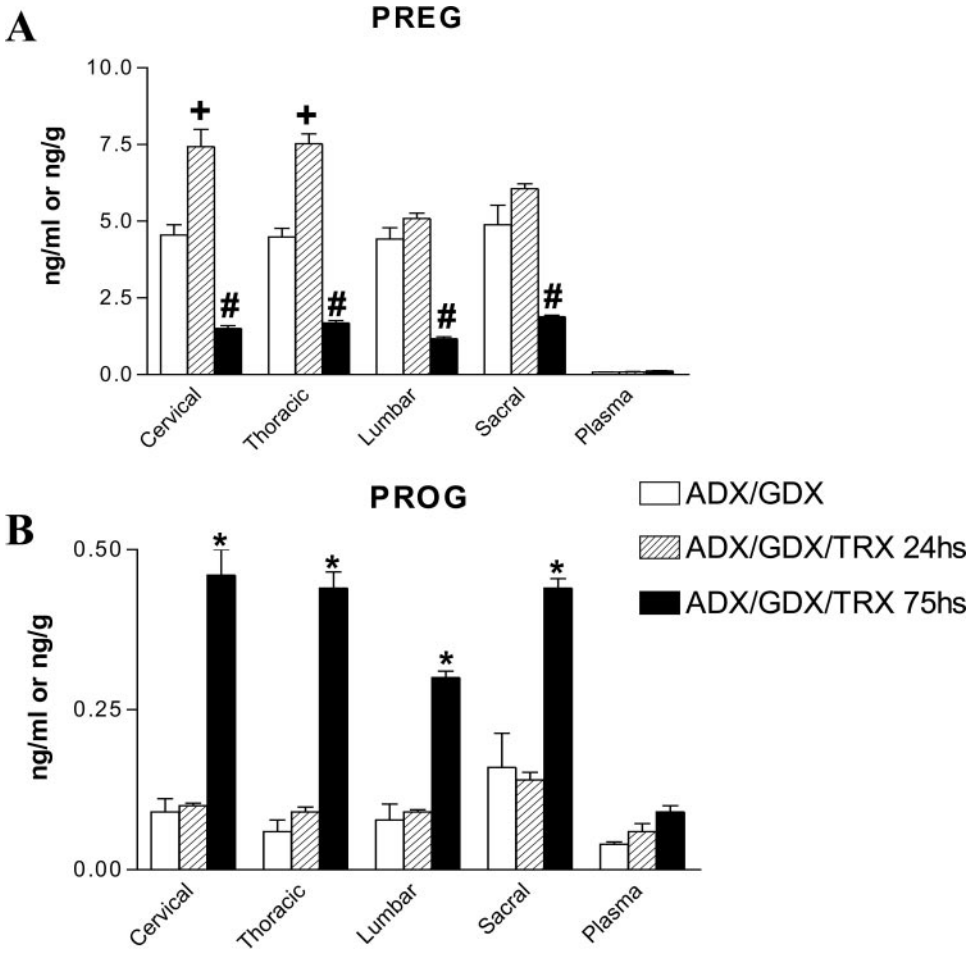

FIG. 6. Effect of SC transection on PREG and PROG concentrations in cervical, thoracic, lumbar, and sacral segments of SC and in plasma of ADX and GDX male rats. Analysis by GC/MS 24 and 75 h after SC transection. Data represent mean ± SEM (n = 4 rats per group). Statistical analysis by two-way ANOVA followed by Bonferroni posttest demonstrated increased PREG levels at 24 h after injury (+, $P < 0.001$ *vs.* ADX/GDX) and decreased levels after 75 h (#, $P < 0.01$ *vs.* ADX/GDX) (A), and no significant change of PROG levels at 24 h after injury and increased levels of PROG 75 h after transection (*, $P < 0.01$ *vs.* ADX/GDX) (B).

3α,5α-THPROG is expressed in SC, indicating a potential local synthesis of these steroids.

After SCI, there was an increase in PREG, PROG, 5α-DHPROG, and 3α,5α-THPROG levels (up to 2- to 3-fold depending on the segment analyzed). For example, increase in PREG levels was more substantial in SC segments distal from the lesion site than in the lumbar region, the segment just below the lesion; Indeed, after SCI, PREG levels represented respectively 271, 190, 163, and 188% of basal levels in the cervical, thoracic, lumbar, and sacral segments of SC. Furthermore, PROG levels increased in the regions distal from the lesion site, whereas there was no increase in the lumbar segment, a region just below the lesion. It is unlucky that it is due to the damage of the tissue but rather to region-specific changes in neurosteroidogenesis. Indeed, in the lumbar region, PROG seems to be actively metabolized to 5α-DHPROG then to 3α,5α-THPROG because an increase in the levels of these metabolites was observed after SCI. The SC may be adapting local steroid synthesis to the needs created by the injury. This increase in progestin levels is likely due to a local synthesis in the SC rather than to an uptake from the circulation. Indeed, the increase in SC steroid was not accompanied by significant changes in plasma levels. A local synthesis of PREG and PROG was further supported by the results from ADX/GDX/TRX rats. In the absence of steroidogenic glands, injury first resulted in increased PREG levels, followed by an increase in PROG levels with a concomitant decrease in the PREG level in SC. The local synthesis of 5α-DHPROG and 3α,5α-THPROG was confirmed by the results from TRX and PROG-treated rats, which showed much higher levels of these steroids in SC than in plasma. Our results have also shown an induction of 3β,5α-THPROG formation in SC when the levels of the precursors PROG and 5α-DHPROG reached high levels (PROG-treated group). The local synthesis of 3β,5α-THPROG in the SC is supported by the observation that this steroid is barely detectable in the plasma.

A previous report suggested an increase in neurosteroid metabolism after brain and SC injuries. When PREG-sulfate was infused 1, 3, or 7 d after fluid percussion of SC at T7–T8, a time-dependant increase in the levels of PROG, 5α-DHPROG, and 3α,5α-THPROG was observed in the proximal perifocal site of injury (28) However, in this study, there was no demonstration of *de novo* synthesis of neurosteroids in the absence of gonads and adrenals, of the metabolism of endogenous neurosteroids, and no information concerning plasma steroid levels.

After SCI, the increase in neurosteroid content in SC was not accompanied by a significant increase in steroidogenic enzymes expression. Thus, the observed local increase in neurosteroids may be due to an increase in steroidogenic enzymes activities and/or an up-regulation of the proteins

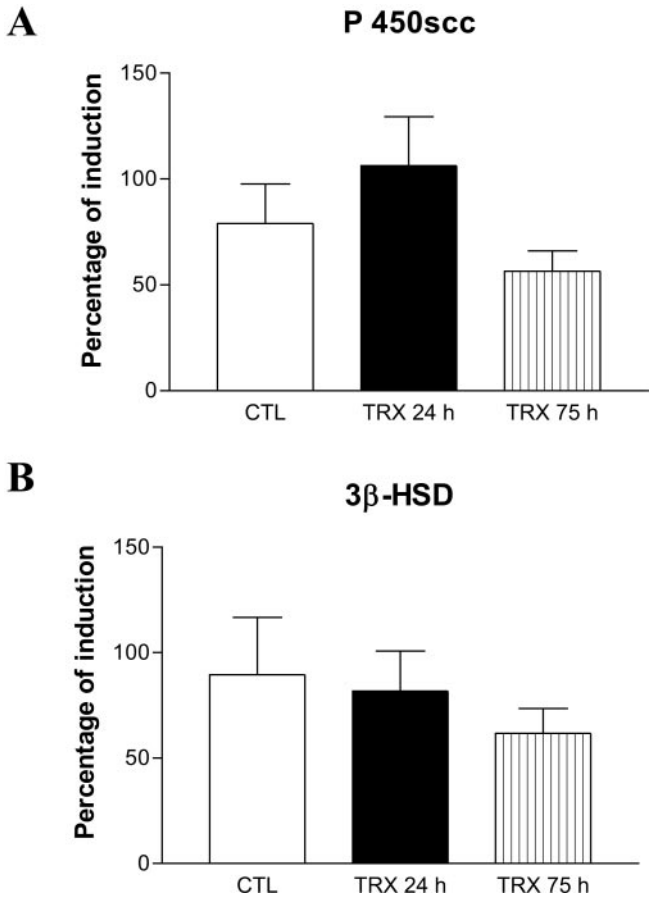

FIG. 7. Effect of SC transection on P450scc and 3β-HSD mRNA expression in the lumbar segment of SC of rat. Analysis by quantitative real-time PCR 24 and 75 h after transection. Data represent mean of the percentage of induction ± SEM (n = 11 rats per group). Statistical comparisons by one-way ANOVA followed by Newman-Keuls test show no significant difference between groups.

involved in the intra mitochondrial trafficking of cholesterol, the first step in steroidogenesis, such as StAR and PBR. Indeed, PBR and StAR expressions have been shown to be up-regulated in different models of nervous system injuries. StAR mRNA and protein levels were acutely and transiently increased in the hippocampus after excitotoxic brain injury induced by the administration of kainic acid (29). After peripheral nerve injury, PBR expression increases in small dorsal root ganglia sensory neurons (30) and in sciatic nerve (31, 32). Furthermore, traumatic brain injury leads to increased expression of PBR (33). Further studies are needed to investigate whether SCI leads to a stimulation of steroidogenic enzymes activities and/or up-regulation of StAR and PBR.

The increase in neurosteroid concentration in SC after injury may present a pivotal role in the capacity of SC to adverse consequences of injury. Indeed, there is emerging evidence that PROG and 3α,5α-THPROG can protect neurons after injury and in particular against glutamate excitotoxicity in different models including SC neurons (34–39). Glutamate is toxic for neurons (40, 41) and for oligodendrocytes (42, 43) at the elevated concentrations it attains after SCI (40, 44). Furthermore, agents that block glutamate receptors improve functional outcomes and reduce damage to traumatized SC tissue (30, 45–50). Thus, release of glutamate clearly contributes to secondary damage after SCI. In addition, it has been shown that NMDA resulted in a dose-dependent increase in the concentration of PREG in rat hippocampal neurons (51). Therefore, this cascade of events might be hypothesized: SCI results in high concentrations of glutamate, which increase neurosteroid synthesis. In turn, neurosteroids decrease the excitotoxicity to glutamate.

Local expression of steroidogenic and steroidogenic-inactivating enzymes has a key role in steroid hormone action (52–54). Enzymes metabolizing a single chemical group, such as hydroxyl or ketone, on the steroid, can either activate or inactivate it. This constitutes an economical on-off switch for regulating steroid hormone action. Selective expression in peripheral organs of enzymes that catalyze this on-off switch is an essential part of paracrine, autocrine, and intracrine mechanisms for regulating steroid response (55), which, together with the expression of steroid receptors in target tissues, provide a flexible mechanism for regulating diverse physiological responses.

High levels of PROG, 5α-DHPROG, 3α,5α-THPROG, and 3β,5α-THPROG are measured in SC 75 h after transection and PROG treatment. These results suggest that the local reduction of PROG in SC may be of particular relevance after TRX and PROG treatment as indicated by the increased 5α-DHPROG to PROG ratio in the SC *vs.* plasma. This observation raises the question of the role of the reduction of PROG after SCI.

A tissue expresses isoenzymes to increase the diversity of responses available to metabolic challenges. SC expresses both type 1 and type 2 5α-reductase isoenzymes. Our results showed a good correlation between the levels of the product 5α-DHPROG and the substrate PROG in SC. Indeed, in control animals, low but significant levels of both steroids were detected, in ADX/GDX animals, when levels of PROG are minimal, there was no production of 5α-DHPROG, and after PROG treatment, there was a high production of 5α-DHPROG. The type 2 isoenzyme of 5α-reductase has apparent Km values for steroid substrates in the nanomolar range, whereas the type 1 has a micromolar affinities (56). If we take into account the levels of PROG present in SC, it can be speculated that, in control rats, the type 2 is the mainly active enzyme, whereas in TRX and PROG-treated rats, both isoenzymes may contribute to the 5α-DHPROG synthesis. Matsui *et al.* (57) have shown that type 2 isoenzyme of 5α-reductase expression was induced in the hippocampus of female mice by PROG. A PROG regulatory element was identified as a highly related sequence to the consensus progesterone response element (PRE)/androgen response element (ARE) sequence in the 5α-reductase 2 promoter (57). Further experiments are needed to demonstrate whether PROG can enhance expression and/or activity of type 1 and 2 isoenzymes of 5α-reductase in SC of TRX and PROG-treated rats.

Reduced metabolites of several steroids interact with $GABA_A$ receptors (58, 59). However, 5α-pregnane steroids also have been shown to interact with the nuclear PROG receptors (60, 61). The high rate of conversion of PROG to reduced metabolites in SC may provide a cross-talk mechanism between membrane and genomic hormonal events. It

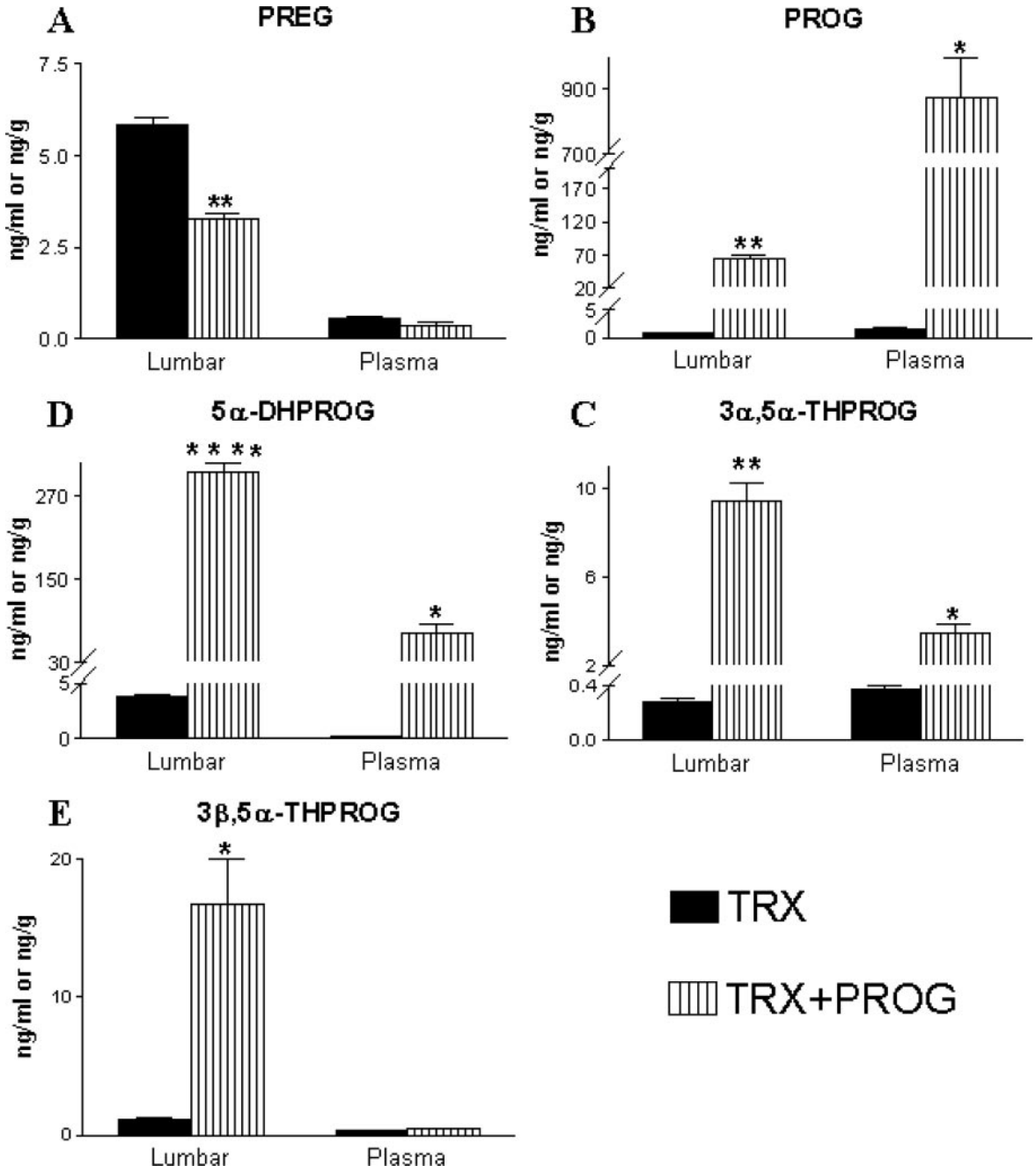

FIG. 8. Effect of SC transection and systemic PROG treatment on PREG, PROG, 5α-DHPROG, 3α,5α-THPROG, and 3β,5α-THPROG concentrations in the lumbar segment of SC and plasma of adult male rats. Data represent mean ± SEM (n = 5 rats per group). Statistical analysis using Student's *t* test demonstrated increased levels of PROG and its metabolites in both plasma and SC. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ *vs.* TRX. TRX+PROG, TRX and PROG treatment.

is not known which of these steroids are effectively the mediators of the PROG response observed *in vivo* (12–15, 22).

The discussion remains open on the possible mechanisms underlying the neuroprotective effects observed *in vivo* after PROG treatment. Progestins may have a beneficial influence after SCI by inhibiting neuronal excitotoxicity through a positive modulation of $GABA_A$ receptors and by direct or indirect trophic effect on neuron and glia by regulating important functional genes. Multiple mechanisms can account for PROG effects in the SC. PROG can act *per se* to modulate the transcription of specific genes after binding to the nuclear receptor PR, which has been detected by immunohistochemistry and RT-PCR (22, 62). In addition, PROG may have membrane effects after binding to the putative PROG membrane receptor 25-Dx, which is also expressed in rat SC and is up regulated by PROG in the injured SC (22). The metabolite 5α-DHPROG may be of high physiological importance because 1) it can bind and activate the PROG receptor (60) and 2) it is the substrate for the 3α-HSD and 3β-hydroxysteroid oxidoreductase enzymes leading respectively to 3α,5α-THPROG and

3β,5α-THPROG formation. Functional analyses indicated that 3α,5α-THPROG promotes neuron survival (38, 39). In mature neurons, 3α,5α-THPROG is well known as a positive allosteric modulator of the $GABA_A$ receptor to increase chloride influx, thereby hyperpolarizing the neuronal membrane potential and decreasing neuron excitability (63–65). In addition, 3α,5α-THPROG has been shown to regulate the proliferation of neural progenitors in dose-dependent manner (66, 67). After SCI and PROG treatment, high levels of 3β,5α-THPROG have been detected in SC. Several reports showed that this 3β-isomer of 3α,5α-THPROG has no effect *per se in vivo* or *in vitro* (68–71). However several recent studies have shown that 3β,5α-THPROG may antagonize the effects of 3α,5α-THPROG (19, 70, 72).

Our results suggest that progestins may play a critical role in the capacity of the SC to respond to injury and demonstrate that the exogenous treatment by PROG results in high tissue levels of both PROG and its reduced metabolites, pointing to several possible mechanisms of actions. These observations thus contribute to better understanding the role of neurosteroids after SCI.

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