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3β-HYDROXYSTEROID DEHYDROGENASE EXPRESSION IN RAT SPINAL CORD

H. COIRINI,[a,b] M. GOUÉZOU,[a] P. LIERE,[a] B. DELESPIERRE,[a] A. PIANOS,[a] B. EYCHENNE,[a] M. SCHUMACHER[a] and R. GUENNOUN[a]*

[a]INSERM U488, 80, rue du Général Leclerc, 94276 Bicêtre, France

[b]Laboratorio de Neurobiología, Instituto de Biología y Medicina Experimental, Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

Abstract—In adult male rats, 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3β-HSD) expressing cells were identified in the spinal cord from the cervical to the sacral segments. An *in situ* hybridization study, using an oligonucleotide common to the four known isoforms of rat 3β-HSD, revealed its mRNA in gray matter. Measurements of optical densities in autoradiograms showed the following regional distribution: dorsal horn (layers I–III) > central canal (layer X) ≥ ventral horn (layers VIII–IX) > ventral funiculus = lateral funiculus. At the cellular level, the number of grains was higher on the large motoneurons than on small neurons of the dorsal horn, but the grain density per cell was similar. Further evidence for the expression of 3β-HSD in the spinal cord was obtained by western blot analysis, which revealed an immunoreactive protein of ≈45 kDa in the dorsal and ventral parts of the spinal cord. Castration and adrenalectomy did not influence the expression of 3β-HSD mRNA and protein. Gas chromatography/mass spectrometry measurements showed higher levels of pregnenolone and progesterone in the spinal cord than in the plasma. After castration and adrenalectomy, their levels remained elevated in the spinal cord, suggesting that these neurosteroids may be synthesized locally.

The wide distribution of 3β-HSD, and the high levels of pregnenolone and progesterone in the spinal cord even after castration and adrenalectomy, strongly suggest a potential endogenous production of progesterone and an important signalling function of this steroid in the spinal cord.

Key words: neurosteroids, 3β-hydroxysteroid dehydrogenase, spinal cord, *in situ* hybridization, progesterone.

There is growing evidence that progesterone (PROG) may be considered as a neuroprotective and neuroregenerative steroid in both the peripheral nervous system and CNS (Schumacher et al., 2000; Stein, 2001). In a contusion model of the rat spinal cord, PROG was shown to reduce the size of the lesion and to promote neuronal survival and functional recovery (Thomas et al., 1999). The specific mechanisms by which PROG promotes morphological and functional recovery are not well understood, however, some of its effects may be mediated by the regulation of gene expression via classical PROG receptors. Indeed, using immunocytochemistry, we have shown that neurons from ventral horn lamina IX, glial cells in gray and white matter and ependymal cells contain PROG receptors (Labombarda et al., 2000b). Additional mechanisms of PROG action may involve protection of neurons against glutamate and a reduction of lipid peroxidation (Roof et al., 1997). In a previous study, Labombarda et al. have shown that PROG modulates the expression of two astrocyte proteins, NADPH-diaphorase and glial fibrillary acidic protein in rat spinal cord. Interestingly, NADPH-diaphorase activity became sensitive to PROG only in response to injury (Labombarda et al., 2000a). Other effects of PROG in spinal cord have been reported. These include changes in nociception by the modulation of dynorphin levels, activation of opiate receptors and enhancement of the antinociceptive effect of muscimol (Beyer, 1994; Dawson-Basoa and Gintzler, 1996; Medina et al., 1993).

The synthesis of PROG from pregnenolone (PREG) is catalyzed by the 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD) enzyme, which is highly expressed in the classical steroidogenic glands, i.e. testis, ovary, adrenal, and placenta, as well as in peripheral tissues, such as liver and skin (Mason, 1993). Four different isoforms of rat 3β-HSD cDNAs have been characterized and their expression has been shown to be tissue specific (Simard et al., 1993; Zhao et al., 1990, 1991). In several brain

*Corresponding author. Tel.: +33-1 49 59 18 80; fax: +33-1 45 21 19 40.

E-mail address: guennoun@kb.inserm.fr (R. Guennoun).

Abbreviations: ADX, adrenalectomized; ANOVA, analysis of variance; 3β,5β-THA, 3β,5β-tetrahydroandrostanedione; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase; CORT, corticosterone; GC/MS, gas chromatography/mass spectrometry; GDX, gonadectomized; HFB, heptafluorobutyric acid anhydrid; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; PREG, pregnenolone; PROG, progesterone; R.O.D., relative optical density; SDS, sodium dodecyl sulfate.

regions, the expression of both 3β-HSD protein and its mRNA has been reported (Dupont et al., 1994; Guennoun et al., 1995, 1997; Sanne and Krueger, 1995). The presence of 3β-HSD has also been demonstrated in neurons and Schwann cells of dorsal root ganglia and in sciatic nerve (Koenig et al., 1995; Guennoun et al., 1997; Robert et al., 2001).

3β-HSD mRNA has been detected by reverse transcription-polymerase chain reaction (RT-PCR) in the rat spinal cord (Sanne and Krueger, 1995). In this study, we have investigated the regional and cellular expression of 3β-HSD mRNA by *in situ* hybridization and the presence of the protein by western blot analysis in the spinal cord. We have also analyzed the effect of castration and adrenalectomy on 3β-HSD expression and on the levels of PROG and its precursor PREG by gas chromatography/mass spectrometry (GC/MS).

EXPERIMENTAL PROCEDURES

Experimental animals

Male Sprague–Dawley rats (200–250 g; Iffa Credo, France) were housed under standard laboratory conditions with a 12-h light/dark cycle and food and water *ad libitum*. Animals used were intact, or gonadectomized (GDX) and adrenalectomized (ADX). Surgical procedures were conducted under ketamine anesthesia. The removal of the testis was done 18 days before adrenalectomy and the animals were killed 3 days after removal of the adrenal glands. The last three days, the GDX/ADX animals received isotonic saline as drinking fluid. Sham-operated animals were used as controls. Tissues were always sampled between 11:00 and 13:00 h. All procedures concerning animal care and use were carried out in accordance with the European Community Council Directive (86/609/EEC).

Tissue preparation

For in situ hybridization. Adult male rats ($n = 5$) were anaesthetized with ketamine and perfused through the heart with phosphate buffer followed by 1% paraformaldehyde. The spinal cords were removed by dorsal laminectomy, post-fixed by immersion in the same fixative for 1 h and transferred to a solution of 15% sucrose in buffer phosphate where they remained overnight. Spinal cords were then sectioned into four regions (cervical, thoracic, lumbar and sacral), frozen on powered dry ice and stored at −70°C. Coronal sections (10 μm) of each of the four different regions (six to nine sections/slide) were mounted on gelatin-subbed slides and stored at −70°C until use. For the GDX/ADX and sham-operated animals ($n = 5$/group), only the lumbar region was studied using the same procedure as described above.

For western blot and steroid analysis. Animals were killed by decapitation, adrenal glands, when they were available, and spinal cords were collected. Western blot analysis was done first in whole spinal cord of control animals ($n = 3$) or in the dorsal and ventral region of the spinal cord, obtained by a longitudinal cut ($n = 5$). For sham-operated and GDX/ADX animals, the lumbar region was used ($n = 4$–6). The concentration of the steroids was measured in lumbar spinal cord and plasma from another set of control and GDX/ADX animals ($n = 5$/group). Truncal blood was collected on heparinized tubes. After removal, tissues were rapidly frozen on liquid nitrogen and stored at −70°C until processing. Plasma was obtained using regular laboratory's techniques no later than 2 h after blood collection and stored at −20°C.

In situ hybridization studies

A 40-base synthetic oligonucleotide for 3β-HSD$_{I–IV}$, 5′-GAT GCT CCC ATT GGC TGC CAG CAC TGC CTT CTC GGC CAT C-3′ (nucleotides 690–651 according to the numbering of the rat 3β-HSD type I) which is complementary to a sequence common to each of the four known 3β-HSD isoforms was selected. The sequence was verified using BLAST search of EMBL and GenBank databases to ensure that there was no homology with other mRNAs. The 3β-HSD$_{I–IV}$ oligonucleotide was labeled with [α^{35}S]dATP (1000 mCi/mmol, Amersham, Les Ulis, France) to a specific activity of 2×10^9 c.p.m./μg using terminal deoxynucleotidyltransferase (Amersham). Spinal cord sections (two slides/animal/region) were incubated in the presence of the ^{35}S-labeled probe according to previously described methods (Guennoun et al., 1995). In brief, 0.25 ng of labeled probe dissolved in the hybridization buffer [20 mM Na_2PO_4, 200 mM dithiothreitol in 2× saline sodium citrate (SSC), containing 50% deionized formamide, 10% dextran sulfate, 5% sarcosyl, 1% Denhardt's solution, 500 μg/ml denaturated salmon sperm DNA and 250 μg/ml yeast tRNA] was applied on the tissue sections. In order to verify the specificity of the hybridization signal, *in situ* hybridization was carried alongside with 500-fold excess of non-radio-labeled probe (cold probe competition). The non-specific hybridization was determined using one slide/animal/region. In addition, this oligonucleotide has already been used for a northern blot analysis and it hybridized only to the 3β-HSD transcripts (Guennoun et al., 1995). Hybridization proceeded overnight at 40°C. The following day, sections were rinsed several times using SCC buffer (1× and 0.1×), dehydrated and opposed to X-ray films (Kodak Biomax MR) for 7 days. Then slides were coated with Ilford K5 emulsion, exposed for 2 months and developed. Sections were stained with Toluidine Blue. The impressions generated on the films and the number of grains deposited on the sections were quantified using a computer assisted image analysis system (Rasband and Bright, 1995). Relative optical densities (R.O.D.) were calculated as the average from three to five different autoradiograms. The value for each animal was considered as the mean of the specific expression from 12–18 spinal cord sections. For the cellular level, different areas of several sections from the same spinal cord region were digitized and analyzed. An average of 150 neurons for the dorsal horn and 30–60 motoneurons of the ventral horn per slide were analyzed (two slides/animal/region). Means values for each region, were calculated as the average from five animals.

Western blot analysis

Adrenals and spinal cord (whole or divided in dorsal and ventral parts by a longitudinal cut) were homogenized in a lysing solution [1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 0.8 mM phenylmethylsulphonyl-fluoride in phosphate-buffered saline (PBS)]. Homogenates were centrifugated at $1500 \times g$ for 15 min. The supernatant containing the proteins was subjected to electrophoresis on 10% polyacrylamide gel. Tissue samples (100 μg protein/50 μl/well) and a buffer sample containing 0.05 M Tris (pH 6.8), 2% SDS, 10% glycerol, and 8 mM ethylenediaminetetra-acetate (EDTA) were boiled for 3 min in the presence of 2-mercaptoethanol and then loaded on the gel. After protein transfer, the nitrocellulose filter was incubated in blocking solution (10% non-fat dried milk powder in PBS containing 0.1% Tween-20) at room temperature for 1 h and rinsed in PBS. The filter was cut to separate the different lanes in order to allow different incubations: with the pre-immune serum antibody (1:100 in PBS), the antibody alone [a rabbit polyclonal anti-3β-HSD antibody described in Guennoun et al. (1995), 1:100 in PBS] or the antibody (1:100) preadsorbed with 150 μg/ml of the peptide. The membranes were washed in PBS, incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) for 30 min, rinsed in PBS Tween-20, and finally incubated in avidin–biotin–peroxidase complex (Vector Laboratories). After 30 min,

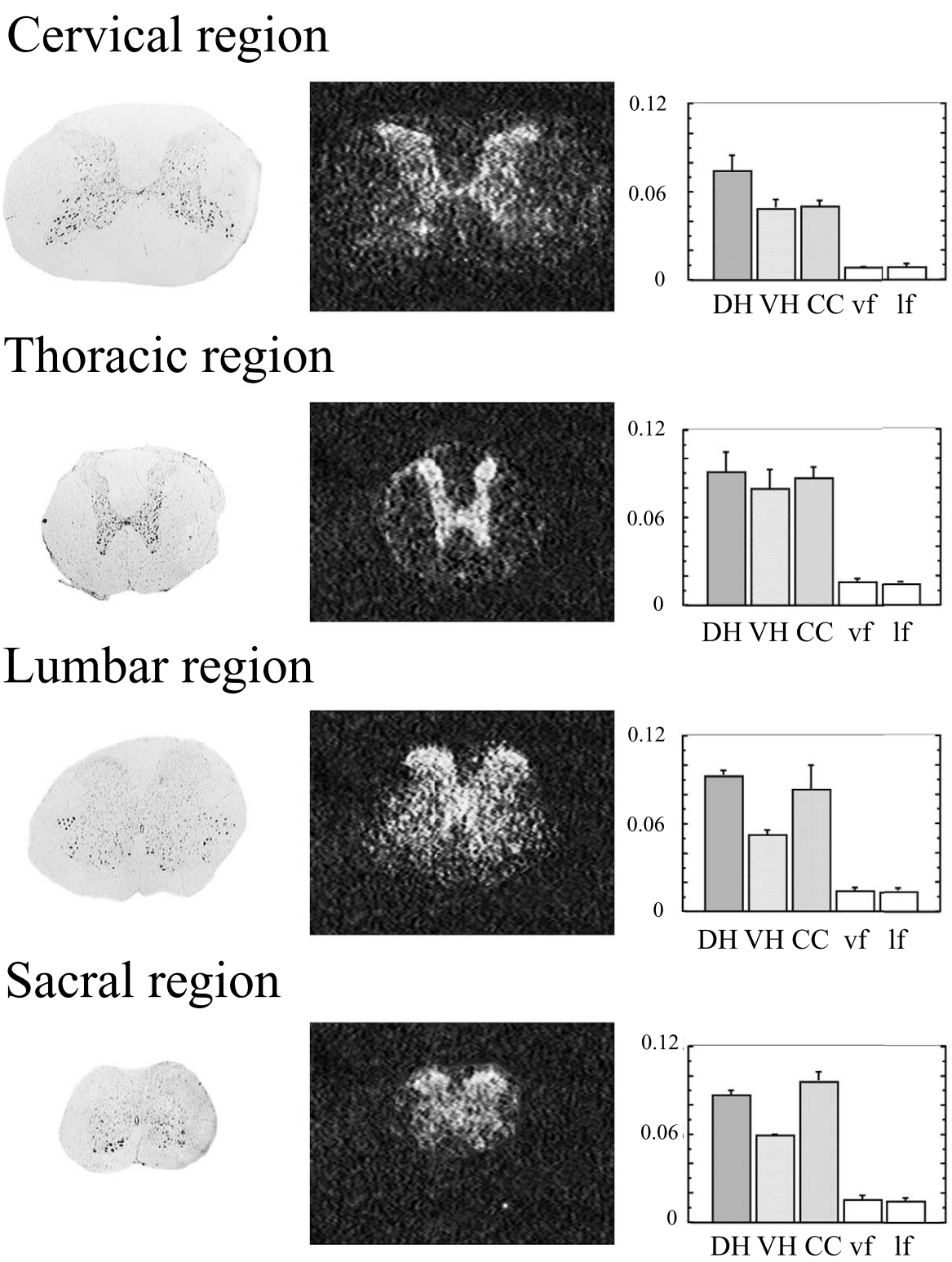

Fig. 1. Regional distribution of 3β-HSD mRNA expression in the rat spinal cord. Representative histological sections (bright field) and their corresponding autoradiograms (dark field) from *in situ* hybridization studies in the spinal cord with 3β-HSD probe. R.O.D. were determined using a computer assisted image analysis system, and are shown at the right. Similar patterns of mRNA expression were observed in the four regions. Relatively higher R.O.D. values were found on the gray matter compared to the white matter. R.O.D. values showed the following distribution: dorsal horn (DH, layers I–III) > central canal (CC, layer X) ≥ ventral horn (VH, layers VIII–IX) ≫ ventral funiculus (vf) = lateral funiculus (lf).

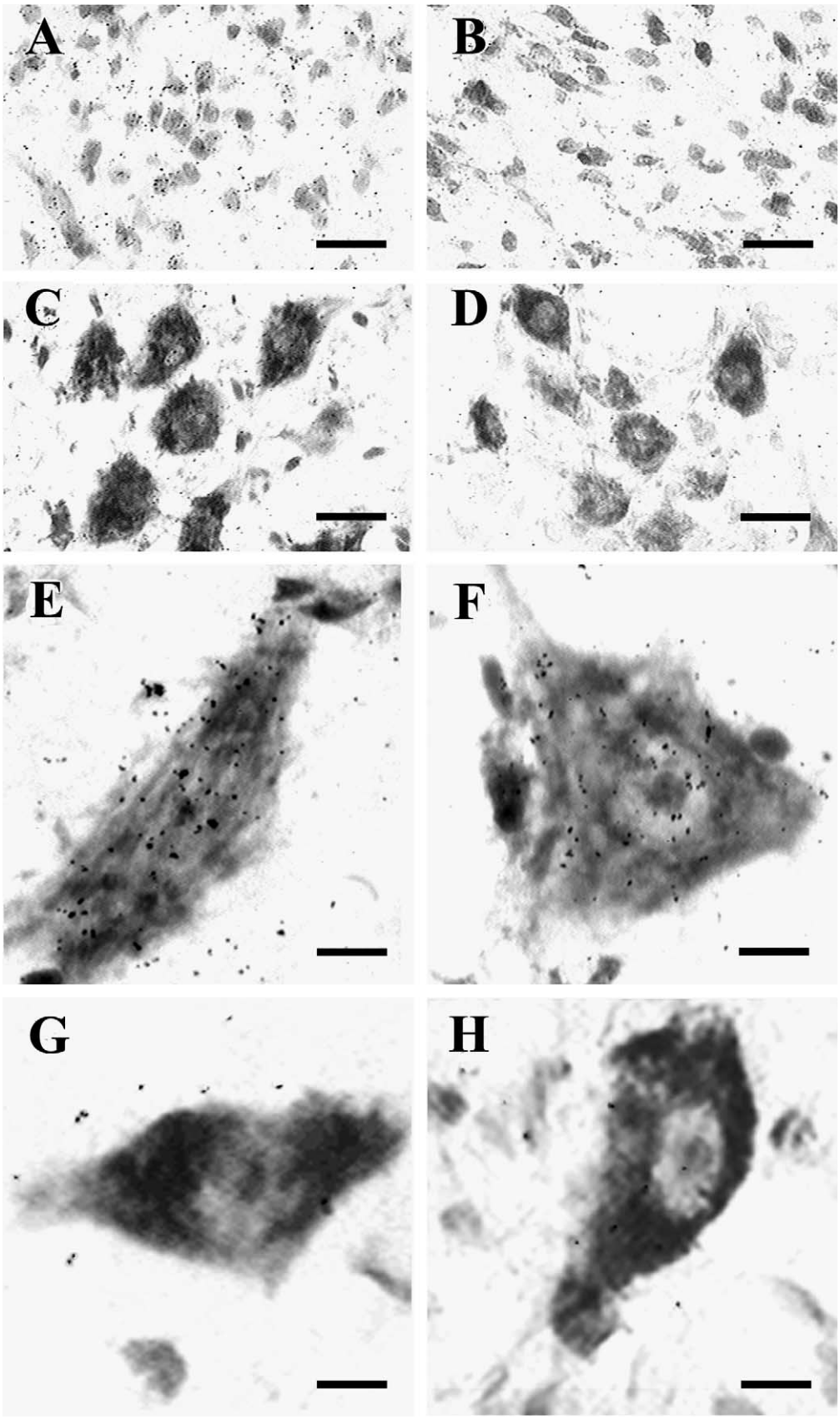

Fig. 2.

blots were washed in PBS and the peroxidase activity was revealed using 4-chloronaphthol in the presence of H_2O_2.

Tissue and plasma steroid levels

Steroid levels in the lumbar spinal cord and plasma were determined by GC/MS (Liere et al., 2000). In brief, samples (50–70 mg tissue or 2 ml plasma) were purified by solid phase extraction on IST C_{18} minicolumns (De Wang et al., 1997) and submitted to high-performance liquid chromatography (HPLC). PREG, PROG, and 3β,5β-tetrahydroandrostanedione (3β,5β-THA) as the internal standard, were collected in a time scale of 10–29 min and corticosterone (CORT) in a time scale of 35–40 min with a 202 model Gilson fraction collector. The steroids from the HPLC fractions were derivatized using heptafluorobutyric acid anhydride (HFB) as reagent. Derivatized samples and calibration solutions were injected with an AS800 autosampler in a gas GC 8000 Top chromatograph (Carlo Erba, Milan, Italy) coupled to an Automass mass spectrometer (model 150, Finnigan Automass, Argenteuil, France). GC was performed in the splitless mode with a 1-min splitless time. The mass spectrometer was operated in the electron impact mode with an emission current of 2000 μA and with an ionization energy of 70 eV. Ion identification was performed in the full scan mode in the mass/charge (*m*/*z*) range of 50–550 Da. Quantification was done in the single-ion monitoring modes, only the *m*/*z* 298 Da (PREG–HFB), 510 Da (PROG–HFB) and 486 Da (3β,5β-THA–HFB) diagnostic ions, were detected in the line scale of elution of each steroid.

Statistical analysis

Results are expressed as the mean ± S.E.M. For the levels of 3β-HSD mRNA expression in the spinal cord (film analysis), statistical differences were evaluated by two-factor analysis of variance (ANOVA) (factor 1: spinal cord region, factor 2: spinal cord layer). Significant differences for each layer among the different spinal cord regions as well as for gray and white matter were evaluated by one-factor ANOVA followed by Scheffe's test. Statistical analysis for the steroid levels in the plasma and the tissue were assessed by two-factor ANOVA (factor 1: treatment, factor 2: location), followed by one-factor ANOVA using Scheffe's test at the significance level of 5%. Differences between two means were analyzed by unpaired or paired, two-tailed *t*-test.

RESULTS

The expression of 3β-HSD mRNA was studied throughout the rat spinal cord. Figure 1 shows representative Toluidine-stained sections of the four different regions, their corresponding autoradiograms and quantitative analysis of optical densities measured on films opposed to the sections.

A strong hybridization signal was detected throughout the spinal cord with the [^{35}S]dATP-labeled oligonucleotide 3β-$HSD_{I–IV}$. A lower signal was observed in the white matter (ventral funiculus and lateral funiculus) with respect to gray matter. A 500-fold excess of unlabeled homologous oligonucleotide completely abolished the hybridization signal (not shown). Measurements of the R.O.D. of the autoradiograms (data from three to five animals/region) showed the following regional distribution throughout the spinal cord from the cervical to the sacral segment: dorsal horn (layers I–III) > central canal (layer X) ≥ ventral horn (layers VIII–IX) > ventral funiculus = lateral funiculus. Two-factor ANOVA (region/layer) followed by Scheffe's test, showed a significant effect on both variables: $F_{region}(3,65) = 79.0$; $F_{layer}(4,65) = 93.4$; $P \leq 0.0001$. Low levels of R.O.D. were determined in white matter (ventral funiculus or lateral funiculus) with respect to gray matter ($P < 0.005$ by one-way ANOVA). Statistical analyses of the mRNA expression showed significant differences on layers VIII–IX (ventral horn) between thoracic and other regions ($T = 0.078 \pm 0.015 > S = 0.058 \pm 0.002$; $L = 0.052 \pm 0.004$; $C = 0.047 \pm 0.006$; ($F(3,12) = 12.192$; $P = 0.0006$, one-way ANOVA followed by Scheffe's test). For the central canal (layer X) significant differences were found between cervical and both thoracic and sacral regions ($C = 0.051 \pm 0.004 < T = 0.086 \pm 0.008$ or $S = 0.095 \pm 0.007$; $F(3,13) = 6.048$; $P = 0.0083$).

After emulsion development, the spinal cord sections were further analyzed at the microscopic level. Representative images of the silver grains deposited on layer II of the dorsal horn (A, B) and on motoneurons of the ventral horn (C, D, E, F, G, H) are shown in Fig. 2. Many small-sized neurons (Fig. 2A) and large motoneurons (Fig. 2C) were labeled with the 3β-HSD oligonucleotide. Higher magnification in Fig. 2E and F showed the heavy labeling on motoneurons. The specificity of the hybridization signal was shown by performing displacement experiments with a 500-fold excess of unlabeled homologous oligonucleotide in adjacent sections. Only background was seen after competition (Fig. 2B, D, G, H).

Analysis of the number of grains per cell (Table 1) showed higher amount of grains on motoneurons than in the neurons of the dorsal horn in the four regions of the spinal cord. However, when the density of grains per cell was calculated, there were no significant differences between the two types of neurons (Table 1).

The expression of the 3β-HSD protein in the spinal cord was assessed by western blot analysis (Fig. 3). Using a polyclonal antibody raised against a synthetic peptide common to the four known 3β-HSD isoforms (Guennoun et al., 1995), we have detected a 45-kDa protein on total spinal cord homogenates. When the spinal cord was divided into dorsal and ventral regions by a longitudinal cut, similar amounts of proteins were detected by immunoblot in both regions [R.O.D. dorsal horn = 24.24 ± 4.45; R.O.D. ventral horn = 24.02 ± 4.06 ($n = 5$); paired *t*-test]. Control assays for the antibody specificity for the 3β-HSD protein in the whole spinal cord and adrenal gland showed the disappearance of

Fig. 2. Cellular distribution of 3β-HSD mRNA expression in the rat spinal cord. Representative images at the cellular level from a lumbar spinal cord section hybridized with ^{35}S-labeled specific 3β-HSD oligonucleotide probe. (A) Silver grains on small-sized neurons of the layer II of the dorsal horn. (C) Silver grains on motoneurons of the ventral horn. (E, F) Higher magnifications showing the labeling on the motoneurons. (B, D, G, H) Control of specificity of the probe. Significant reduced amount of silver grains was observed on neuronal cells when 500-fold excess of unlabeled probe was added in the hybridization reaction. Scale bar = 100 μm (A–D); 250 μm (E–H).

Table 1. Quantification of the 3β-HSD mRNA signal on neurons of the spinal cord after hybridization

Region	Number of grains		Grain density	
	Neurons (layer II)	Motoneurons (layers VIII–IX)	Neurons (layer II)	Motoneurons (layers VIII–IX)
Cervical	5 ± 3	30 ± 14	7.73 ± 3.42	8.69 ± 4.49
Thoracic	6 ± 3	22 ± 10	8.69 ± 3.69	12.30 ± 4.90
Lumbar	9 ± 4	57 ± 23	7.06 ± 3.46	7.33 ± 5.91
Sacral	9 ± 4	43 ± 21	8.05 ± 3.83	11.01 ± 6.23

The number of grains per cell was higher on motoneurons than in the neurons of the laminae II. However the density of grains per cell was similar between the four different regions of the spinal cord. The number of silver grains observed on the white matter was always just above the background.

the specific band when the antibody was preadsorbed with 150 μg/ml of the immunization peptide (data not shown).

The regulation of the 3β-HSD mRNA by gonadectomy and adrenalectomy was examined in one representative region: the lumbar region of the spinal cord. Since grain counting analysis showed no significant differences among the spinal cord regions, we decided to study the effect of castration and adrenalectomy only in one region. The lumbar region was chosen because there are sexual dimorphic nuclei in this region (Breedlove and Arnold, 1980) and different studies reported the effect of gonadal and adrenal hormones in this region (Gonzalez et al., 1999; Labombarda et al., 2000a, 2002). Sham-operated animals served as controls. Analysis of autoradiograms showed no significant differences in the levels of R.O.D. (0.29 ± 0.01 for controls; 0.28 ± 0.02 for GDX/ADX; unpaired *t*-test), either on whole spinal cord sections, or when dorsal and ventral horns were analyzed separately (Fig. 4A; data not shown). Western blot analysis of the 3β-HSD protein content from another set of animals showed no difference in the amount of protein detected in both groups [Fig. 4B; R.O.D. control = 10.57 ± 1.91 ($n = 6$); R.O.D. GDX/ADX = 11.27 ± 3.26 ($n = 4$), unpaired *t*-test].

Since the absence of changes in the expression and amount of the enzyme present in the lumbar spinal cord may or may not correlate with the enzyme activity, we have measured the levels of the substrate and product of the 3β-HSD in both experimental groups (Table 2). The steroid levels were determined by GC/MS in the lumbar region of the spinal cord and the plasma. Circulating levels of CORT were quantified by the same method. Intact animals showed a relative elevated value of CORT (278.5 ng/ml) similar to those observed following moderate stressor, however, the levels on GDX/ADX were not detectable. PREG and PROG levels were higher in spinal cord than in plasma [PREG: $F_{\text{location}}(1,16) = 68.2$ / PROG: F_{location} $(1,16) = 101.4$; $P \leq 0.0001$; two-factor ANOVA (treatment/location)]. After castration and adrenalectomy, there was a significant decrease in the levels of PREG (80%) and PROG (92%) in the plasma (PREG: $F(1,8) = 8.0$; $P = 0.015$/ PROG: $F(1,8) = 12.0$; $P = 0.008$, one-factor ANOVA) while significant amount of both steroids remained present in the tissue. Statistical analysis of steroid levels for each individual animal showed a significant correlation between the levels of PREG and PROG in the spinal cord (control: $P < 0.05$; $r_2 = 0.78$, GDX/ADX: $P < 0.05$; $r_2 = 0.86$; Fig. 5). A similar correlation was found in plasma, but only in the control animals ($P < 0.001$; $r_2 = 0.98$). In contrast, when spinal cord levels were compared to plasma levels, no significant correlation was observed in any experimental group either for PREG or PROG.

DISCUSSION

The present study demonstrates the expression of 3β-HSD in the rat spinal cord at the message and protein levels. It is the first detailed analysis of the neuroanatomical distribution of 3β-HSD mRNA in this part of the nervous system. Analysis of R.O.D. on films opposed to spinal cord sections showed that 3β-HSD mRNA is expressed throughout the spinal cord, within the cervical, thoracic, lumbar and sacral regions. 3β-HSD mRNA was more highly expressed in gray matter than in white matter and in dorsal horns when compared to ventral horns. However, the latter difference was only due to a higher density of neuronal cell bodies in the dorsal region.

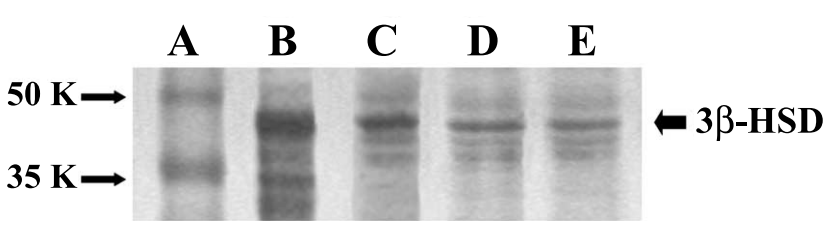

Fig. 3. Western blot analysis of the 3β-HSD protein in the spinal cord. Proteins extracted from homogenates of adrenal gland (B), whole spinal cord (C), dorsal spinal cord (D) and ventral spinal cord (E) from intact male rats (100 μg) were separated on 10% polyacrylamide gel and then transferred to nitrocellulose filters. The blot was incubated with the specific 3β-HSD antibody. Positions of the molecular markers (kDa) are indicated in the left part of the figure (A).

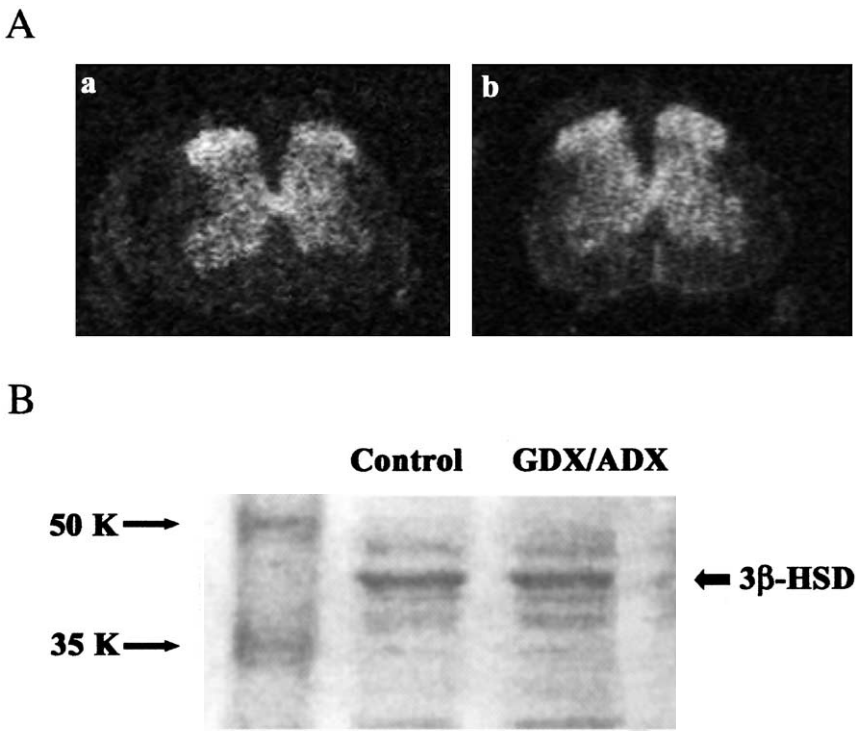

Fig. 4. Effect of GDX/ADX on spinal cord 3β-HSD expression. (A) *In situ* hybridization analysis. Similar pattern of mRNA expression was observed in control and GDX/ADX lumbar spinal cord. (B) Western blot analysis. The intensity of the 3β-HSD immunoreactive protein was similar in control and GDX/ADX lumbar spinal cord homogenates.

To understand the role of PROG in spinal cord functions, it is essential to identify the cells involved in its synthesis. It has been generally accepted that glial cells play a major role in neurosteroid formation and metabolism. In contrast to glial cells, the concept of neurosteroidogenesis in neurons has been uncertain. Synthesis of PROG was thought to mainly occur in glial cells. Conversion of PREG to PROG was demonstrated in mixed cultures of oligodendrocytes and astrocytes (Jung-Testas et al., 1989), in purified astrocytes where 3β-HSD may be regulated by cell density (Akwa et al., 1993) and in Schwann cells from sciatic nerve (Robert et al., 2001). However, *in vivo* studies using *in situ* hybridization demonstrated the presence of 3β-HSD mRNA in neurons of several brain regions (Guennoun et al., 1995) and in rat sensory neurons of dorsal root ganglia (Guennoun et al., 1997). 3β-HSD mRNA was detected in neurons of olfactory bulb, caudate putamen, accumbens nucleus, olfactory tubercle, cortex, thalamus, hypothalamus, hippocampus, medial habenular nucleus and cerebellum. In the olfactory bulb, 3β-HSD mRNA was detected in granular, mitral, and glomerular layers. In the hippocampus, 3β-HSD mRNA was detected in granule cells in the dentate gyrus and pyramidal cells of CA3 and CA4. In the cerebellum, 3β-HSD mRNA was detected in the granular and molecular layers (Guennoun et al.,

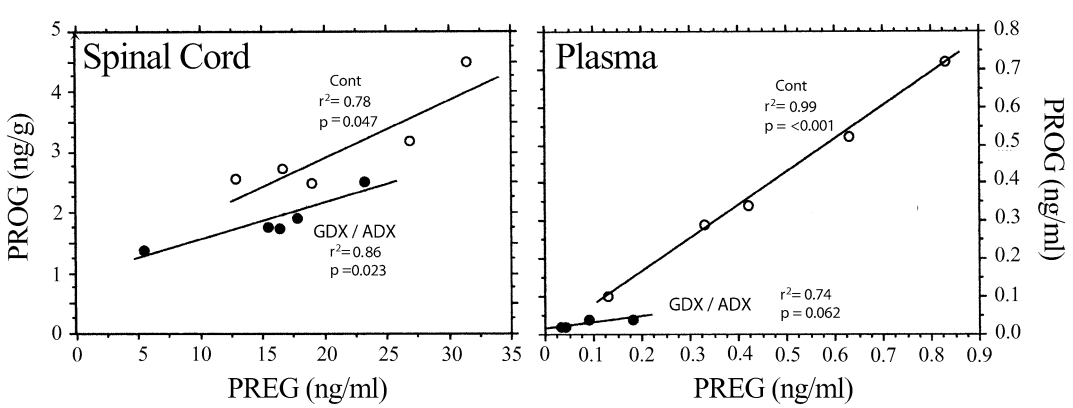

Fig. 5. Correlation between the levels of PREG and PROG in control and castrated/ADX rats. Regression plots between the amount of PROG vs. PREG in the lumbar spinal cord and plasma from sham-operated (Cont) and GDX/ADX, male rats. Steroid levels were determined using GC/MS ($n = 5$ animals/group). Each point represents the values corresponding to one animal. Significant correlation was found on steroid levels present in the spinal cord of both groups and in the plasma of control animals.

Table 2. Neurosteroid levels in the plasma and lumbar spinal cord of intact and castrated/ADX rats

Steroid		PREG	PROG
Plasma	control	0.468 ± 0.121	0.394 ± 0.105
	GDX/ADX	0.096 ± 0.028*	0.028 ± 0.005*
Spinal cord	control	21.35 ± 3.42	3.098 ± 0.371
	GDX/ADX	16.27 ± 2.90	1.642 ± 0.188*

The levels of PREG and PROG in plasma and the lumbar spinal cord of GDX/ADX and sham-operated animals (control) were determined by GC/MS. Steroids were extracted with methanol and derivatized with HFB, after purification and fractionation by HPLC. These products were analyzed by GC/MS using selected ion monitoring. Data represent the mean ± S.E.M. of five animals/group and they are expressed as ng/g of tissue or ng/ml of plasma. PREG and PROG levels were higher in spinal cord than in plasma (PREG: $F(1,16) = 68.2$/PROG: $F(1,16) = 101.4$; $P \leq 0.0001$; two-factor ANOVA). *$P < 0.05$ compared to control one-factor ANOVA.

1995). Different studies have demonstrated that Purkinje cells actively synthesize several neurosteroids, including PROG, de novo from cholesterol (for review, see Tsutsui et al., 2000). Recently, Sakamoto et al. have shown that PROG acts directly on Purkinje cells to promote dendritic growth and synaptogenesis during the neonatal period (Sakamoto et al., 2001). In another study, the expression of 3β-HSD protein was extensively analyzed in different regions of the frog CNS by immunocytochemistry. As in the rodent brain, 3β-HSD was detected in neurons (Mensah-Nyagan et al., 1994). In DRG we have previously shown that sensory neurons express 3β-HSD mRNA and that they are able to convert PREG to PROG. The conversion of PREG to PROG by sensory neurons is positively regulated by a diffusible factor produced by glial cells (Guennoun et al., 1997). 3β-HSD mRNA is mainly expressed in neurons within spinal cord, like for the brain and DRG. Thus PROG remaining present after GDX/ADX may be mainly of neuronal origin.

It has been previously shown that significant steroid levels remain in other neural tissues after the removal of peripheral steroids by procedures such as ADX and castration (Corpéchot et al., 1981, 1983). We measured relatively high levels of PREG and PROG in the spinal cord compared to plasma. In addition, the amount of PREG in the spinal cord after castration and adrenalectomy was not essentially different from that present in control tissue. Even when the levels of PROG in the GDX/ADX tissue were significantly lower than in sham-operated animals, the levels of PREG and PROG also showed a significant correlation. These observations may suggest that the spinal cord is able to synthesize PROG in both experimental conditions depending upon the availability of the substrate for 3β-HSD enzyme. The absence of changes in the expression of 3β-HSD mRNA and protein after gonadectomy and adrenalectomy together with the high levels of PREG and PROG in the spinal cord support the idea of the existence of neurosteroid synthesis in the spinal cord where PREG may be used as substrate for the 3β-HSD.

CONCLUSION

Our results demonstrate for the first time the regional and cellular distribution of 3β-HSD in the spinal cord, showing that it has the potential to synthesize PROG locally. The expression of this enzyme seems to be constitutive, is evenly distributed and is not affected by the removal of the steroidogenic glands. We have also presented indirect evidence for the local production of neurosteroids after castration and adrenalectomy. The capacity of the spinal cord to locally synthesize steroids may provide autonomous control to this tissue which can adjust its production and metabolism to local requirements at physiological or pathological states. Treatment with PROG has been shown to exert beneficial and neuroprotective effects in the injured spinal cord (Labombarda et al., 2000a, 2002; Thomas et al., 1999). Whether the locally produced PROG has an effect on motoneurons remained to be demonstrated. Therefore, future attention should be focused on local PROG synthesis and its effects on spinal cord motoneurons.

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