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Effects of injury and progesterone treatment on progesterone receptor and progesterone binding protein 25-Dx expression in the rat spinal cord

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Abstract

Progesterone provides neuroprotection after spinal cord injury, but the molecular mechanisms involved in this effect are not completely understood. In this work, expression of two binding proteins for progesterone was studied in intact and injured rat spinal cord: the classical intracellular progesterone receptor (PR) and 25-Dx, a recently discovered progesterone membrane binding site. RT-PCR was employed to determine their relative mRNA levels, whereas cellular localization and relative protein levels were investigated by immunocytochemistry. We observed that spinal cord PR mRNA was not up-regulated by estrogen in contrast to what is observed in many brain areas and in the uterus, but was abundant as it amounted to a third of that measured in the estradiol-stimulated uterus. In male rats with complete spinal cord transection, levels of PR mRNA were significantly decreased, while those of 25-Dx mRNA remained unchanged with respect to control animals. When spinal cord-injured animals received progesterone treatment during 72 h, PR mRNA levels were not affected and remained low, whereas 25-Dx mRNA levels were significantly increased. Immunostaining of PR showed its intracellular localization in both neurons and glial cells, whereas 25-Dx immunoreactivity was localized to cell membranes of dorsal horn and central canal neurons. As the two binding proteins for progesterone differ with respect to their response to lesion, their regulation by progesterone, their cellular and subcellular localizations, their functions may differ under normal and pathological conditions. These observations point to a novel and potentially important role of the progesterone binding protein 25-Dx after injury of the nervous system and suggest that the neuroprotective effects of progesterone may not necessarily be mediated by the classical progesterone receptor but may involve distinct membrane binding sites.

Keywords: 25-Dx, neuroprotection, progesterone receptor, spinal cord injury, steroids.

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It is now well documented that steroid hormones provide neuroprotection after injury of the central nervous system (CNS) (De Nicola 1993; Schumacher *et al*. 2000). Multiple studies have focused on the potential beneficial effects of glucocorticoids after spinal cord trauma (Bracken *et al*. 1990; Hall 1993; Gonzalez *et al*. 1996, 1999). However, the list of neuroprotective steroids has increased over the past years. Thus, progestagens (Stein 2001), androgens (Jones *et al*. 2001) and estrogens (Garcia-Segura *et al*. 2001; Wise *et al*. 2001; Betz and Coester 1990) have been shown to decrease the extent of brain injury and to promote neuronal survival.

Protective and trophic effects of progesterone (PROG) have been documented in the rat spinal cord after traumatic injury or during disease. Thus, PROG promoted neurological recovery after spinal cord contusion. Rats treated with PROG showed better locomotor activity and less tissue and white

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Abbreviations used: ALS, amytropic lateral sclerosis; EB, estradiol benzoate; GFP, green fluorescent protein; HRE, hormone-response element; IZAg, inner zone antigen; PBS, phosphate-buffered saline; PR, progesterone receptor; PROG, progesterone; TRX, transection; VEMA, ventral midline antigen.

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matter damage than control animals (Thomas *et al*. 1999). In the Wobbler mouse, a model for degenerative motoneuron diseases such as amytropic lateral sclerosis (ALS) (Duchen and Strick 1968; Price *et al*. 1994), PROG has recently been shown to rescue motoneurons from degeneration, based on histological abnormalities and on α3 and β1 subunit Na,K-ATPase mRNA levels (Gonzalez Deniselle *et al*. 2002a; Gonzalez Deniselle *et al*. 2003).

The mechanisms by which PROG exerts these protective effects are not well known and may involve protection against excitotoxicity (Ogata *et al*. 1993), inhibition of free radical-induced lipid membrane peroxidation (Roof *et al*. 1997) and selective regulation of gene expression. Indeed, we have previously shown that specific neural and glial markers in the rat spinal cord become sensitive to PROG in response to injury. Thus, PROG did not affect NADPH-diaphorase active astrocytes in the intact spinal cord, but increased their number after spinal cord transection (Labombarda *et al*. 2000a). In spinal neurons, PROG treatment of transected rats restored expression of the choline acetyltransferase and of two subunits (α3 and β1) of the neuronal Na,K-ATPase and increased GAP-43 mRNA levels. However, in intact spinal cord, PROG had no effect on these three markers (Labombarda *et al*. 2002).

According to the classical model, PROG regulates the expression of its target genes after binding to a selective intracellular receptor, which belongs to a superfamily of zinc finger transcription factors (Evans 1988). After hormone binding, the activated PR binds to specific hormone-response elements (HRE), generally located in the promoter region of target genes, and interacts with multiple coregulator proteins (McKenna *et al*. 1999; Beato and Klug 2000). The expression of the PR undergoes multiple regulations. In hypothalamic and limbic structures containing estrogen receptors, the PR is induced by estradiol, whereas in other brain regions including the cerebral cortex, septum and cerebellum, the PR is not affected by estrogenic stimulation (MacLusky and McEwen 1978; Parsons *et al*. 1982; Romano *et al*. 1989). In most reproductive tissues, including the brain regions involved in the control of reproduction, PROG down-regulates the expression of its own receptor (Savouret *et al*. 1989).

The presence of an estrogen-insensitive PR has been recently demonstrated in the rat spinal cord by immunocytochemistry (Labombarda *et al*. 2000b). Neurons from ventral horn Lamina IX, glial cells in gray and white matter and ependymal cells are PR-positive. Whereas in cells of the pituitary gland and uterus the PR is exclusively localized within the nucleus, it is also present in the cytoplasm and in processes of spinal neurons and glial cells (Labombarda *et al*. 2000b).

Recently, a putative membrane receptor for PROG (mPR) has been cloned from porcine vascular smooth muscle cells (Falkenstein *et al*. 1996, 1999) and later in humans (Gerdes *et al*. 1998). Using [^{3}H]PROG as radioligand, binding studies show high-affinity and low affinity binding sites in microsomal preparations of porcine liver membranes with an apparent K_{d2} of 11 nM and an apparent K_{d2} of 286 nM. In solubilized fractions the high affinity binding sites are present at an apparent K_{d2} of 69 nM. The purified fraction, showing the maximum specific PROG-binding activity, corresponds to two major polypeptides of apparent molecular masses of 28 and 56 kDa (Meyer *et al*. 1996). The expression of mPR cDNA in CHO cells lead to an increase in microsomal PROG binding. In addition, incubation of spermatozoa with an antibody raised against the recombinant *Escherichia coli* mPR suppresses the rapid PROG-initiated Ca^{2+} increase in sperm (Falkenstein *et al*. 1999). The rat homolog of this membrane site mPR, cloned from liver, encodes a 223 amino acid peptide and has been named 25-Dx (Selmin *et al*. 1996). Its mRNA is also expressed in many regions of the rat brain, and a green fluorescent protein (GFP) fusion construct has allowed to demonstrate the membrane localization of 25-Dx in a neuronal cell line (Krebs *et al*. 2000). Highest levels of 25-Dx are found in hypothalamic and limbic structures, suggesting a role of this membrane binding site of PROG in the control of reproductive functions. In the hypothalamus of female rats, 25-Dx expression is increased by estrogen and repressed by PROG (Krebs *et al*. 2000). The functions of 25-Dx in other brain regions such as the cerebral cortex, hippocampus, striatum and cerebellum are unknown, as are the functional events taking place after PROG binding to 25-Dx. The protein shares sequence homology with the cytokine receptor superfamily (Selmin *et al*. 1996), adrenal inner zone antigen (IZAg) (Raza *et al*. 2001) and the ventral midline antigen (VEMA) cloned from the rat CNS (Runko *et al*. 1999) of hitherto unrelated functions.

In this study, we have investigated the presence and regulation of the PR and of 25-Dx expression in rat spinal cord by RT-PCR and immunocytochemistry. The aim was to describe the response of both PROG binding proteins to injury and hormone treatments. Results suggest that in addition to the classical PR, 25-Dx may play a major role during regenerative processes in the nervous system.

Materials and methods

Animals and surgical procedures

Adult Sprague–Dawley rats (200–250 g) were used. For spinal cord transection (TRX), animals were anesthetized with an intraperitoneal 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 spinal cord lesioned groups. In the last case, after careful laminectomy a complete spinal cord TRX was carried out at thoracic level T10 using the sharp edge of a 25 G needle (Gonzalez *et al*. 1999). Sham-operated controls were laminectomized but the spinal cord left intact. Four experiments were performed. Experiment 1 was planned to study the effect of

estradiol on PR expression in spinal cord. For that, male rats without spinal cord lesion were used. One group received estradiol benzoate (Sigma Aldrich, St Louis, MO, USA) in corn oil (10 μg/rat during 4 days) while the other received vehicle. As positive control of PR, 1 week ovariectomized estrogenized (10 μg/rat during 4 days) female rats were prepared in parallel to take the uterus. Experiment 2 was planned to study the effect of time after lesion on spinal cord PR expression using RT-PCR. To this end, male rats with sham-operation or spinal cord TRX were killed at 6, 24 or 72 h following lesion. Experiment 3 was designed to study the effect of PROG (Proluton, Schering Laboratories, Argentina) treatment on the expression of PR and 25-Dx using RT-PCR and immunohistochemistry. For this purpose, male rats with spinal cord TRX received four injections of vehicle or 4 mg/kg PROG at times 1 h (intraperitonealy), and subcutaneously again at 24, 48 and 72 h post-lesion. This dose of PROG was chosen because it prevents neuronal loss after brain injury (Roof *et al*. 1994; Thomas *et al*. 1999) and modulates motor neuron and glial cell markers after spinal cord injury (Labombarda *et al*. 2000a; Labombarda *et al*. 2002). In experiment 3, two sets of animals were prepared, one for RNA extraction followed by RT-PCR to study the regulation of PR and 25-Dx at the mRNA level. The second set of animals was designed to study the regulation of PR and 25-Dx at the protein level using an immunohistochemical analysis. For the three experiments, the lumbar region of the spinal cord was dissected out for RT-PCR and immunohistochemical analysis. This level was chosen because lesions that destroy descending tracts lead to transynaptic degeneration of motoneurons below the lesion site (Eidelberg *et al*. 1989). Additionally, PROG effects on glial cells and motoneurons in the lumbar region have already been described after transection at T10 (Labombarda *et al*. 2000a, 2002). As positive control of PR expression, uterus of estrogenized females from experiment 1 was used, while for 25-Dx expression, hypothalamus of control male rats from experiment 1 was used. A fourth experiment was designed to check the efficiency of the PROG treatment in experiment 3: One set of animals (CTL, TRX, TRX + PROG) was prepared in order to measure the PROG levels achieved in plasma and the lumbar region of the spinal cord. PROG levels were measured by gas chromatography/mass spectrometry (Liere *et al*. 2000).

Animal studies were carried out following international guidelines, as stated in the Guide for the Use and Care of Laboratory Animals (NIH Guide, Instituto de Medicina y Biología Experimental, Assurance Certificate N A5072-01) and the experiment was approved by the Institute's Animal Care and Use Committee. For post-operative care, animals were caged singly and body temperature was maintained at 37°C by placing the rats under a heating lamp until full recovery from anesthesia. Drinking water was initially provided by direct delivery to the snout until the animals could drink by themselves. The bladder was emptied manually. Analgesic drugs were not given as they may interfere with steroid binding to intracellular receptors (Patel and Rosengren 2001). Rats were kept for a maximum of 72 h after transection or sham surgery.

Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Animals were prepared as described above for experiments 1, 2, and 3. Tissues were sampled, frozen on dry ice and stored at −80°C until use.

RNA extraction. Frozen samples from the lumbar region of the spinal cord (∼ 26 mg, experiments 1, 2 and 3), uterus (experiment 1) or hypothalamus (experiment 1) were ground into powder in a mortar pre-cooled with liquid nitrogen. Total RNA was then extracted using TRIZOL reagent (Life Technologies, Grand Island, NY, USA) 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.

Reverse transcription (RT). Total RNA was subjected to DNAse 1 (Stratagene, La Jolla, CA, USA) 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 (Gibco/BRL) for 90 min at 42°C in the presence of random hexamer primers.

Amplification. An appropriate volume of the cDNA obtained by RT was used in subsequent PCR amplification of PR, 25-Dx and 28 S ribosomal RNA. For PR, a forward primer 5′-ACAGCCTG-CCCCAAGTCTAC-3′ and a reverse primer 5′-CCATCGAGGG-CTCTCATAACT-3′ derived from nucleotides 1565–1586 and 1928–07 of the rat PR cDNA sequence (accession number NM_022847) (Parke-Sarge and Mayo 1994), were used for amplification of a 380-bp fragment. For 25-Dx, primers were: forward, 5′-GGCCATCAACGGCAAGGTGT-3′ and reverse, 5′-AGATATGCTCCACTGACTGC-3′ corresponding to nucleotides 370–389 and 711–692 of the rat 25-Dx (accession number U63315) and of the progesterone receptor membrane component 1 (Pgrmc1) (accession number NM_021766) published sequences (Selmin et al. 1996), they were expected to amplify a cDNA fragment of 341 bp. For 28 S, primers were: forward 5′-AAGC-AGGAGGTGTCAGAAA-3′ and reverse, 5′-TGCCGTATC-GTTCCGCTTG-3′corresponding to nucleotides 7944–7962 and 8315–8297 of the published sequence (accession number V01270) (Subrahmanyam *et al.* 1982); they were expected to amplify a 371-bp fragment. Each PCR reaction contained 200 ng cDNA template, 1 × Taq DNA polymerase buffer, and one of the following concentrations of primers: 0.4 μM for PR, 0.8 μM for 25-Dx, 0.2 μM for 28 S, 200 μM of each dNTP, and 1 Unit of Taq DNA polymerase (ATGC) in a total volume of 50 μL. The conditions of amplifications were: 2 min at 94°C, followed by the appropriate number of cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min After determination of the optimal conditions for PR, 25-Dx and 28 S, 35 cycles, 30 cycles and 15 cycles were chosen, respectively (See Results and Fig. 1 for details). These conditions would allow detection of the messengers remaining in the log phase of amplification. Aliquots of the amplified products and 100 bp DNA ladder were separated on a 1.2% agarose gel and visualized by ethidium bromide staining. The gels were subsequently quantified using Biorad's Image Analysis System and Molecular Analyst for Macintosh software. The relative levels of gene expression were measured by determining the ratio between the products generated from the genes of interest (PR, 25-Dx) and

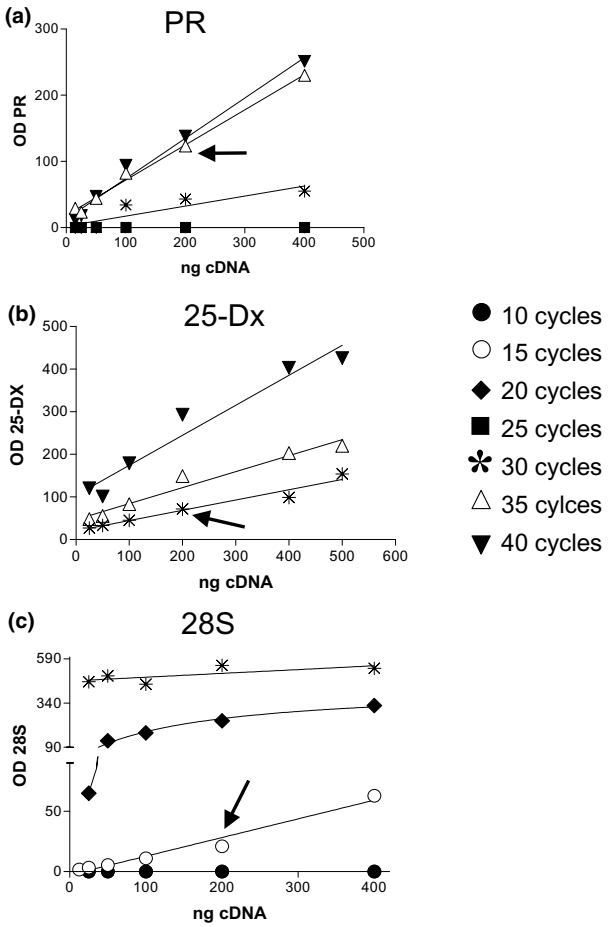

Fig. 1 Validation of the semiquantitative RT-PCR analysis of PROG receptor (PR), 25-Dx and 28S RNA. Different amounts of cDNAs obtained by RT from lumbar spinal cord total RNA were amplified by PCR for 10–40 cycles in the presence of specific primers for PR, 25-Dx and 28S RNA. The number of cycles is denoted by different symbols. (a) PR; (b) 25-Dx; (c) 28S rRNA. Quantification was carried out by Biorad's Image Analysis System and Molecular Analyst for Macintosh software. Arrows show the conditions chosen for the further analysis.

the endogenous internal standard (28 S) in separate reactions (Horikoshi *et al.* 1993).

Immunohistochemistry

For PR immunostaining, a previously published procedure was followed (Labombarda *et al*. 2000b). Anesthetized animals were heart perfused with ice-cold 0.9% NaCl followed by 0.1 M phosphate buffer containing 15% picric acid and 4% paraformaldehyde. Cryostat sections 16-μm thick were rinsed in 0.01 M phosphate buffer containing 0.14 M NaCl and exposed to 0.3% H_2O_2 to block endogenous peroxidase. After preincubation in 10% horse serum during 10 min at 37°C, incubation proceeded overnight at 4°C with a 1 : 100 dilution of an antibody recognizing the B form of PR (KC 146 monoclonal antibody, Dr G. Greene, Ben May Institute for Cancer Research, Chicago, IL, USA) in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 and 1% horse serum. After washing with PBS, sections were incubated with biotinylated secondary antibody (1 : 200 dilution, 60 min) and then ABC complex for 30 min (ABC kit, Vector, CA, USA). The peroxidase activity was revealed using nickel sulfate intensified diaminobenzidine tetrachloride (0.25 mg/mL, Sigma, St Louis, MO, USA) as substrate in the presence of 0.01% H_2O_2 for 8–10 min in the dark. The sections were given a final rinse in PBS, dehydrated in graded ethanols and xylene, and mounted with Permount.

For 25-Dx immunocytochemistry the IZAb antibody was used. This antibody recognizes the rat adrenal Inner Zone antigen (IZAg; Laird *et al.* 1988; Raza *et al*. 2001), identical to the supposed progesterone membrane receptor cloned by Falkenstein *et al*. (1996, 1999). Animals were perfused as described for PR. Paraffin-embedded spinal cords were sectioned at 5 μm and mounted on gelatin-coated slides. Sections were deparaffinized in descending concentrations of xylene and alcohol, washed in phosphate-buffered saline (PBS, 10 mM at pH 7.4) and exposed during 30 min to 0.3% H_2O_2 in methanol to block endogenous peroxidase. After a preincubation with 3% horse serum prepared in PBS during 20 min at room temperature, sections were incubated during 3 h with a 1 : 10 dilution of the monoclonal IZAb antibody prepared in 1% horse serum to block non-specific binding. After a wash with PBS, sections were incubated with biotinylated secondary antibody (1 : 200 dilution, 30 min) and then ABC complex for 30 min (ABC kit, Vector, CA, USA). Subsequent steps, color development and mounting were similar to those described above for PR immunocytochemistry with the exception that nickel sulfate was not employed for intensification.

Number of PR or 25-Dx immunoreactive cells were determined by computer-assisted image analysis in Lamina IX ventral horn or dorsal horn neurons. The cytoarchitectonic division of Steiner and Turner (1972) was used for lamina and cell identification. After location of presumably α-motoneurons (> 900 μm^2) or dorsal horn neurons (< 60–90 μm^2), neuronal density of each section was determined in areas of 3×10^4 μm^2 for both the ventral horn and the dorsal horn. Data corresponding to 6–8 sections per animal ($n =$ 4 animals per group) were combined to give an independent mean, and the animals were used as independent variables (Gonzalez Deniselle *et al*. 2002). Double counting was unlikely because a minimum of 50 μm spacing was kept between adjacent sections. Additionally, PR immunoreaction staining intensity per unit cell area (in μm^2) was also determined. The Optimas Program used in our analysis transforms differences in color intensity of immunopositive cells into gray differences, and results were expressed as the inverse log of gray density per area (ILIGV/area) (Ferrini *et al*. 1995).

Statistical analysis

Data were analyzed by one-way ANOVA, followed by *post hoc* comparisons with the Newman-Keuls test.

Results

Validation of semiquantitative reverse transcriptase polymerase reaction (RT-PCR)

Semi-quantitative RT-PCR analysis was carried out on total RNA extracted from the lumbar region of the spinal cord. In

parallel to amplification of PR and 25-Dx, amplification of rat 28 S rRNA was performed in separate reactions, and used as an internal standard for normalization of the results.

Figure 1 illustrates the validation of the semiquantitative RT-PCR method. Reverse transcription was carried out using 2 μg of total RNA from spinal cord. All total RNA was assumed to be transcribed into cDNAs. Then, PCR amplification for PR, 25-Dx and 28 S rRNA were assayed using different amounts of cDNA (0, 50, 100, 200, 300, 400, 500 ng). For each point, a different number of cycles (range 10–40 cycles) was used to determine the optimal number that would allow detection of the messengers within the log phase of amplification. After quantification of accumulated PCR products, the chosen optimal conditions for the subsequent PCRs were the following: PR, 200 ng of cDNA and 35 cycles (Fig. 1a); 25-Dx, 200 ng and 30 cycles (Fig. 1b); and 28 S rRNA, 200 ng and 15 cycles (Fig. 1c).

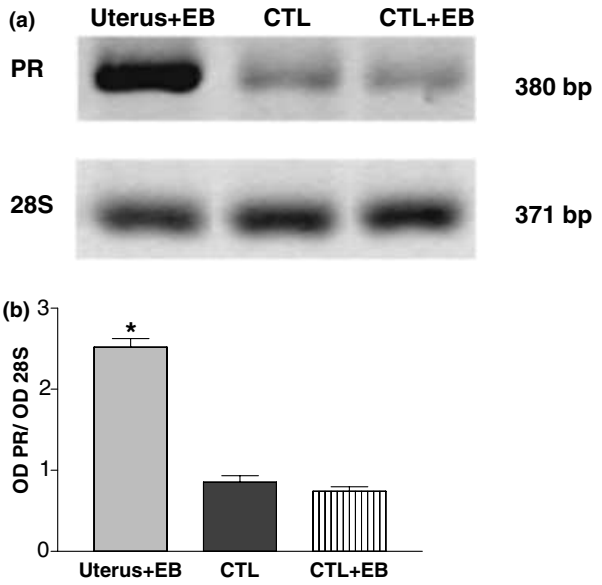

Fig. 2 Expression of PROG receptor (PR) in the rat spinal cord and uterus. (a) Representative gel electrophoretic analysis of RT-PCR products corresponding to PR (upper gel) and 28S RNA (lower gel) in uterus of ovariectomized rats receiving 10 μg estradiol benzoate (EB) during 4 days, in lumbar spinal cord of naive control rats (CTL) and in control rats receiving EB (CTL + EB). (b) Histogram showing PR mRNA expression evaluated by the ratio of PR/28S PCR products in uteri of estrogenized rats and in spinal cord of CTL and CTL + EB groups. PR mRNA levels in spinal cord were a third of the amount found in the estrogen-stimulated uteri (*uterus + EB versus CTL or CTL + EB; $p < 0.001$). PR mRNA expression was similar in CTL and CTL + EB spinal cords. Results represent the mean ± SEM of $n = 4$ (CTL + E2) or 6 (uterus + EB and CTL) determinations per group.

Estradiol effects on PR expression in the spinal cord

Semi-quantitative RT-PCR showed that PR mRNA was expressed in the intact spinal cord of control rats sham operated as described in material and methods for experiment 1. The level of this expression represented 34% ($p < 0.001$) of that observed in the uterus of estrogenized female animals used as a positive control tissue. Treatment with estradiol benzoate (EB) did not change the level of its expression in the spinal cord (cf, CTL versus CTL + EB; Fig. 2). These observations extended our previous results showing no effect of estradiol on PR immunocytochemical staining in the spinal cord (Labombarda *et al*. 2000b).

Time-course of the decrease in PR expression after transection of the spinal cord

Figure 3 shows the temporal profile of PR mRNA expression after spinal cord injury, as described in material and methods for experiment 2. PR expression rapidly declined after the lesion. PR mRNA levels decreased to 34% at 6 h after spinal cord transection (TRX) when compared with control animals (CTL, $p < 0.05$) and remained below control levels at 24 h (66%) and 72 h (55%) ($p < 0.05$).

Progesterone levels in plasma and spinal cord

Measurements of PROG levels by gas chromatography/mass spectrometry of samples from experiment 4 show that low – but nevertheless detectable – steroid levels were present in plasma or spinal cord of control or TRX rats (Table 1). PROG levels, however, were not significantly different in controls versus TRX rats. The PROG treatment as applied in experiments 3 and 4, resulted, respectively, in 925- and 50-fold higher PROG levels in the plasma and the lumbar spinal cord of TRX + PROG group compared with TRX group (Table 1).

Effect of progesterone on PR mRNA in the transected spinal cord

Rats treated with PROG or naive animals were killed 72 h after spinal cord TRX (experiment 3). This time point was chosen based on previous observations of PROG effects on motoneuron markers in the injured spinal cord (Labombarda *et al*. 2002). In agreement of data of Fig. 3, PR mRNA levels represented only 55% of control levels in spinal cord injured animals (Fig. 4, $p < 0.05$). This reduction was not modified by PROG treatment, as levels of PR in TRX + PROG represented 62% ($p < 0.05$) of control levels (CTL, Fig. 4). Thus, this experiment showed that PROG was without effect on the reduced levels of PR mRNA caused by spinal cord TRX.

Effect of progesterone on 25-Dx mRNA expression in the transected spinal cord

The down-regulation of PR mRNA observed after spinal cord injury, raised the question whether a similar response may apply to 25-Dx mRNA. To test this point (experiment 3), expression of the membrane-associated PROG-binding

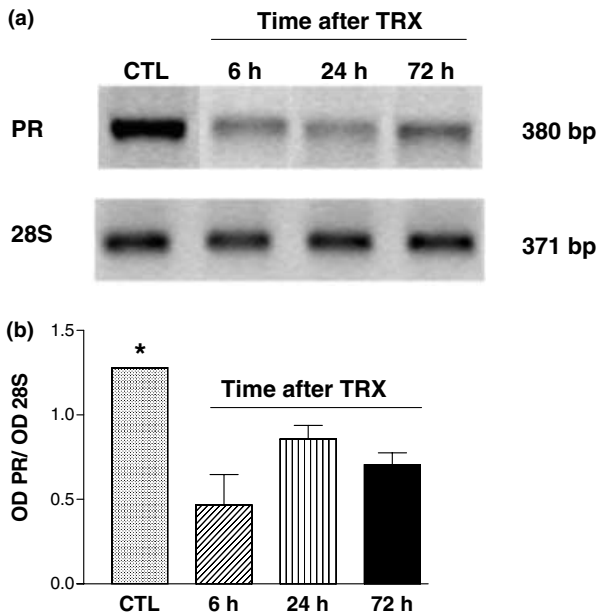

Fig. 3 PR mRNA expression in the control spinal cord and several times after complete spinal cord transection (TRX). (a) Representative gel electrophoretic analysis of RT-PCR products of PR mRNA (upper gel) and 28 S RNA (lower gel) in control (CTL) and 6, 24 and 72 h TRX spinal cords. (b) Quantitative data obtained at different times following TRX. Results represent the mean ± SEM of $n = 4$ (6 and 24 h), 6 (CTL) or 10 (72 h) determinations per group. Statistical analysis using ANOVA and *post hoc* Newman-Keuls test, demonstrated significant reductions of PR mRNA at all times after TRX (* CTL versus 6, 24 and 72 TRX, $p < 0.05$).

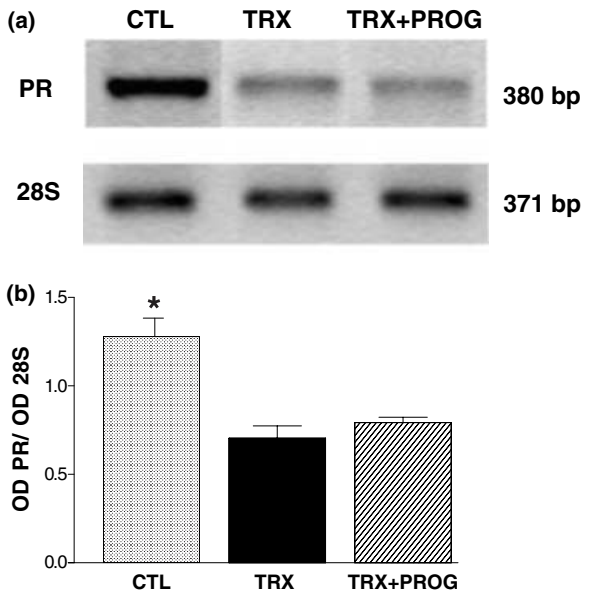

Fig. 4 Effect of PROG treatment of rats with spinal cord transection (TRX) on PR mRNA expression. (a) Representative gel electrophoretic analysis of RT-PCR products corresponding to PR mRNA (upper gel) and 28 S RNA (lower gel) in control (CTL) and 72 h after lesion (TRX) without or with PROG treatment (TRX + PROG). (b) Histogram showing PR mRNA expression evaluated by the ratio of PR/28 S PCR products obtained from CTL, TRX and TRX + PROG groups. Whereas TRX reduced PR mRNA expression (*CTL versus TRX and TRX + PROG, $p < 0.05$), PROG did not change the low expression levels (TRX versus TRX + PROG = NS). Results represent the mean ± SEM of $n = 6$ (CTL), 10 (TRX) and 4 (TRX + PROG) determinations per group.

Table 1 Levels of PROG in plasma and the lumbar spinal cord as determined by GC/MS

Experimental groups	PROG in plasma (ng/mL)	PROG in spinal cord (ng/g)
CTL	1.99 ± 0.69	0.93 ± 0.25
TRX 72 h	1.41 ± 0.71	0.91 ± 0.11
TRX + PROG 72 h	1305 ± 295 **	44 ± 21 *

CTL, control rats; TRX 72 h: spinal cord transected rats killed 72 h after lesion; TRX + PROG 72 h: spinal cord transected rats given 4 mg/kg progesterone at times 1 h (intraperitoneally), and subcutaneously again at 24, 48 and 72 h post-lesion. $N = 4$ animals per group. Statistical analysis: *$p < 0.01$ versus TRX and CTL; **$p < 0.001$ versus TRX and CTL (one-way ANOVA followed by Newman-Keuls test).

protein 25-Dx was analyzed by RT-PCR (Fig. 5). As expected, the hypothalamus of control (CTL) male rats, used as a positive control tissue, expressed high levels of 25-Dx mRNA. In the spinal cord of CTL animals, levels of 25-Dx mRNA represented 26% of those present in the hypothalamus (Fig. 5, $p < 0.001$). In disparity with the profile expression of PR mRNA, no significant changes were observed for 25-Dx mRNA levels at 72 h after injury (Fig. 5, CTL versus TRX, NS). However, and in contrast to data for PR mRNA, PROG up-regulated 25-Dx expression in the injured spinal cord. Thus, in the TRX + PROG group, 25-Dx mRNA was 86% higher than in CTL ($p < 0.001$) and 57% higher than in the TRX only group ($p < 0.001$) (Fig. 5).

Immunocytochemical localization and regulation of PR and 25-Dx proteins in the spinal cord

Immunolocalization of PR. By using the KC 146 monoclonal antibody which recognizes the B form of PR, immunoreactivity was found in large cells, presumably in α-motoneurons located in Lamina IX (Fig. 6a). Staining was prominent in the cytoplasm and in the nucleus. Furthermore, PR immunoreactive glial cells with stellate-like contour or rounded morphology were seen in the white matter (Fig. 6b) which may correspond to astrocytes and oligodendrocytes, respectively. PR immunoreaction was also obtained in the ependymal cells lining the central canal (results not shown). As a positive control for the KC 146 antibody, we used sections of anterior pituitaries. In this case,

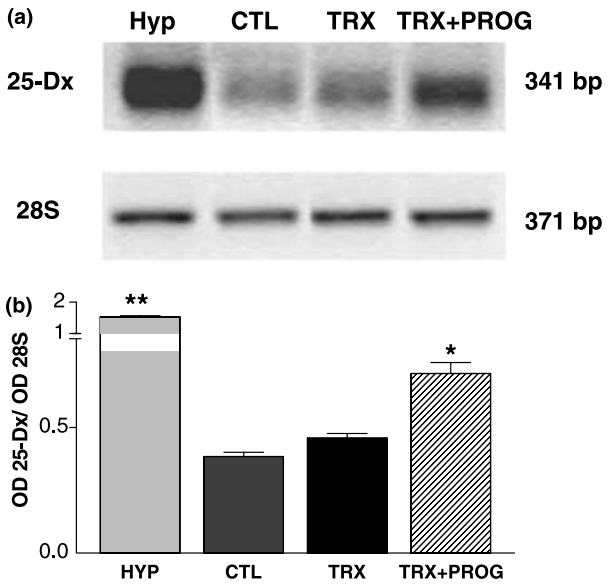

Fig. 5 Effects of transection and PROG treatment on 25-Dx mRNA expression in the rat spinal cord. (a) Representative gel electrophoretic pattern of RT-PCR analysis corresponding to 25-Dx mRNA (upper gel) from hypothalamus (Hyp), and spinal cord groups: CTL, TRX and TRX + PROG (for abbreviations, see legend of Fig. 4). (b) Quantitative histogram obtained from the ratio of 25-Dx/28 S PCR products in hypothalamus (Hyp) and spinal cord of CTL, 72 h TRX and 72 h TRX + PROG groups. Statistical analysis showed that Hyp contained the highest amount of 25-Dx mRNA, compared with all spinal cord groups (**$p < 0.001$). In the TRX + PROG group, 25-Dx mRNA was significantly higher (*$p < 0.001$) than in CTL or TRX groups. Results are the mean ± SEM of $n = 6$ (CTL), 10 (TRX) and 4 (TRX + PROG) observations.

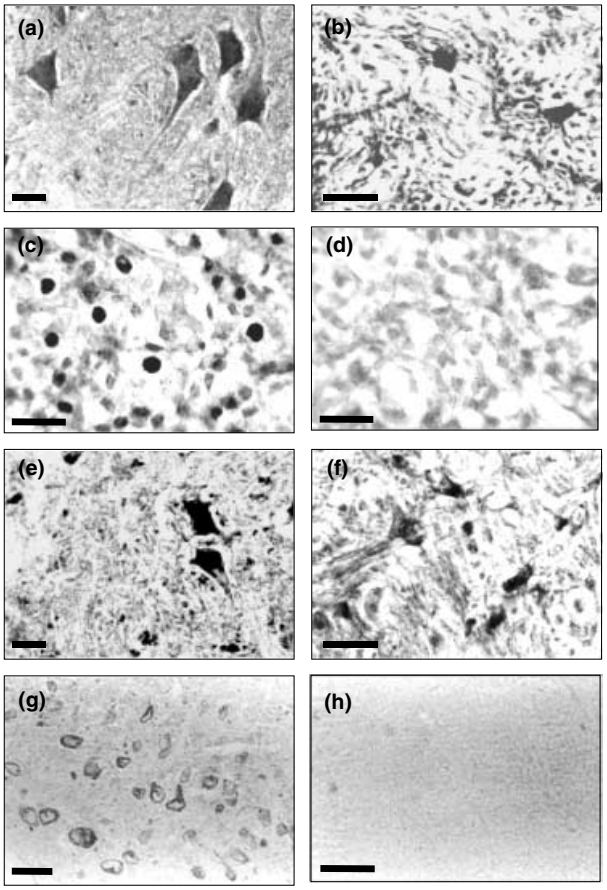

Fig. 6 Profile of PR and 25-Dx like immunoreactivities in spinal cord. Photomicrographs represent staining of PR with the KC-146 antibody in Lamina IX motoneurons (a) and white matter glial cells (b). Staining is concentrated in cytoplasm, nucleus, and some cell processes. In contrast to the localization of PR in the spinal cord, staining with the KC-146 antibody of the anterior pituitary of estrogenized rats showed a strict nuclear localization (c), which was markedly reduced without estrogen treatment (d). Staining with the Let-81 antibody produced a pattern similar to that of KC-146 in motoneurons (e) and glial cells (f). 25-Dx like immunostaining obtained with the IZAb antibody, was confined to dorsal horn neurons (g) and central canal cells (not shown) whereas glial cells were unlabeled (h). Scale bar = 50 μm.

exclusive nuclear staining was obtained in estrogenized rats (Fig. 6c) which was absent in untreated rats (Fig. 6d). A similar staining pattern for the spinal cord was obtained with the Let-81 antibody (a gift of Dr E. Milgrom, Paris, France), which showed strong staining of cytoplasm and nucleus in both motoneurons (Fig. 6e) and glial cells (Fig. 6f).

Regulation of PR expression. The regulation pattern of PR gene expression at the protein level paralleled that observed for mRNA levels. Indeed, transection induced a high decrease in the PR immunostaining intensity of both motoneurons and glial cells. Progesterone treatment did not restore PR expression. The intensity of PR immunostaining in TRX + PROG group was similar to untreated transected animals (TRX). The number of PR immunopositive cells was similar in all the three groups (Fig. 7 and Table 2).

Immunolocalization of 25-Dx. In contrast to the localization of PR immunostaining in neurons and glial cells, the staining with the IZAb antibody which recognizes 25-Dx protein, was exclusively neuronal, showing a preferential staining of the dorsal horn and central canal neurons (Fig. 6g). Staining was prominent in membranes but absent from the nucleus. Glial cells of the white or gray matter were negative for IZAb immunostaining (Fig. 6h). Therefore, cellular as well as subcellular distribution of PR and 25-Dx were quite different. 25-Dx immunoreaction product was confined to membranes of neurons found in sensory regions of the spinal cord, in contrast to a more widespread localization of PR immunoreactivity in neurons, including motoneurons, as well as glial cells.

Regulation of 25-Dx expression. After spinal cord transection the number of IZAb immunolabeled cells decreased by 28.5%. Progesterone treatment increased the number of positive cells. Indeed, the number of positive cells in CTL

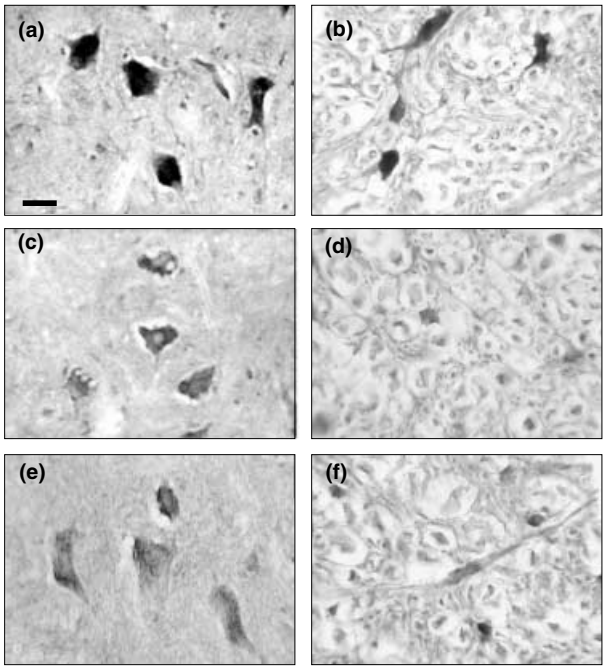

Fig. 7 Effect of transection and progesterone treatment on PR expression in motoneurons (a,c,e) and glial cells (b,d,f). (a) and (b) control motoneurons and glial cells, respectively. The intensity of PR-immunoreactivity was reduced in TRX (c,d) rats compared with CTL rats. The intensity of PR-immunoreactivity of TRX + PROG (e,f) was similar to that of TRX group. Scale bar = 50 μm.

Table 2 Effects of spinal cord transection and progesterone treatment on immunoreaction staining intensity (ILIGV/μm^2) and number of PR-expressing motoneurons in the ventral horn

Experimental groups	Intensity (ILIGV/μm^2) of PR-immunoreactive motoneurons in LIX	Number of PR-immunoreactive motoneurons in LIX
CTL	0.24 ± 0.011	8 ± 2.9
TRX 72h	0.14 ± 0.015*	10 ± 1.87
TRX+PROG 72h	0.13 ± 0.041*	8.3 ± 3.2

Abbreviations as described in the legend to Table 1. $N = 3$ animals per group. Statistical analysis: *$p < 0.01$ TRX 72 h and TRX + PROG 72 h versus CTL (one-way ANOVA followed by Newman-Keuls test).

and TRX + PROG were similar. The intensity of labeling seemed to be in the same order of magnitude for the three groups (Fig. 8 and Table 3). The quantification of intensity of labeling was difficult to perform because of the localization of 25-Dx.

Discussion

In the present study, we demonstrated the expression and differential regulation of the intracellular PR and of the PROG membrane binding protein by semiquantitative RT-PCR and by immunocytochemistry in the rat spinal cord. Staining with specific antibodies also showed differences with respect to their cellular and subcellular localization.

Levels of PR mRNA were elevated in the intact spinal cord, as they amounted to one third of those measured in the estrogen-stimulated uterus. However, they remained

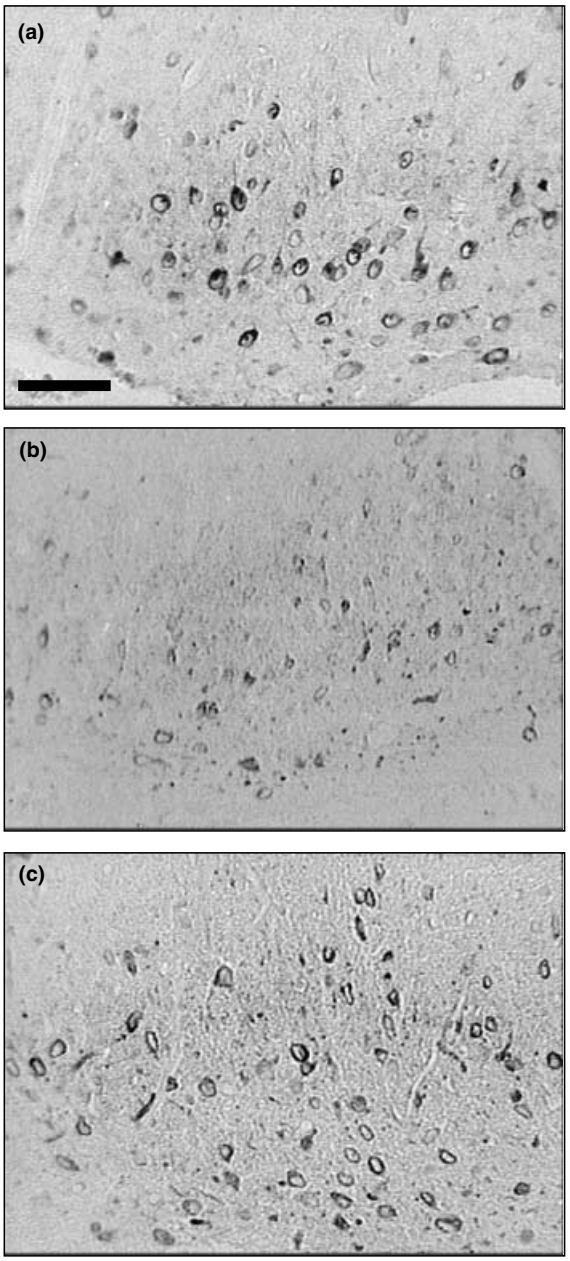

Fig. 8 Effect of transection and progesterone treatment on 25-Dx expression. (a) Control rat; (b) spinal cord TRX; (c) spinal cord TRX plus progesterone. The number of 25-Dx immunoreactive cells decreased after transection (compare a and b). Progesterone treatment restored the number of labeled cells to a level similar to controls (compare a and c). Scale bar = 50 μm.

Table 3 Effects of spinal cord transection and progesterone treatment on the number of 25-Dx immunoreactive cells in the dorsal horn

Experimental groups	Number of 25-Dx immunopositive cells/30μm^2 in dorsal horn
CTL	32 ± 1.52
TRX 72 h	22.90 ± 1.97*
TRX + PROG 72 h	36.63 ± 1.82**

Abbreviations as described in the legend to Table 1. $N = 4$ animals per group. Statistical analysis: *$p < 0.05$ TRX 72 h versus CTL; **$p < 0.001$ TRX + PROG 72 h versus TRX 72 h (one-way ANOVA followed by Newman-Keuls test).

unchanged after estrogen treatment, in agreement with results from a previous study showing that PR-immunoreactivity in the spinal cord is not affected by estradiol (Labombarda *et al.* 2000b). Constitutive expression of PR, which is not regulated by estrogen, has also been described for several brain regions in which ER are expressed at very low levels or absent, as for example the cerebral cortex, septum, caudate putamen, supraoptic nucleus, dentate gyrus, midbrain and cerebellum (MacLusky and McEwen 1978; Parsons *et al.* 1982; Camacho-Arroyo *et al.* 1994).

We demonstrate for the first time the presence of significant amounts of 25-Dx mRNA in the spinal cord, which were however, lower than those found in the hypothalamus, a brain area enriched in this membrane binding site for PROG (Krebs *et al.* 2000). An important observation was the different regulation of PR and 25-Dx mRNA levels by injury and PROG treatment: (i) TRX of the spinal cord produced an important and long-lasting reduction of PR mRNA levels (up to 66%), which could be observed as long as 72 h after the lesion. In contrast, 25-DX mRNA levels were not affected by TRX. In this respect, the behaviors of the PR and of 25-Dx substantially differ from the spinal glucocorticoid receptor, which shows striking up-regulation after spinal cord injury (Gonzalez *et al.* 1990; Yan *et al.* 1999). (ii) After TRX, the administration of PROG did not affect PR mRNA levels, but significantly increased those of 25-Dx.

Histochemically, the spinal cord PR did not present a strict nuclear localization, or estrogen dependency, in contrast of what it was observed here and previously in the anterior pituitary or uterus (Labombarda *et al.* 2000b). The cytoplasmic localization of PR in the spinal cord was also observed with the Let-81 antibody, which stained both motoneurons and glial cells. It may be a peculiarity of the spinal cord to express steroid receptors in cytoplasm and nucleus. This pattern was also shown for the spinal cord glucocorticoid receptor (Ferrini *et al.* 1993). Recently, immunocytochemical localization of estrogen receptor α and β has been reported by another group in perikaryon, nucleus and neurites of lumbar spinal cord motoneurons from rats (Islamov *et al.* 2003)). Using transfection experiments and immunofluorescence analysis of GFP-PRA and B chimeras, Lim *et al.* (1999) have shown that the two forms are differentially distributed between the nucleus and the cytoplasmic compartment, in the unliganded state. Thus, GFP-PRA is predominantly located in the nucleus, whereas GFP-PRB distributes between both the nucleus and the cytoplasm. PRB, the unsubstituted receptor manifests a pattern of localization similar to that of GFP-PRB, indicating that the presence of GFP tag has little effect on the localization of PRB form. Several mechanisms for this differential subcellular distribution of PRA and PRB are suggested (Lim *et al.* 1999). In addition, a nucleocytoplasmic shuttling of the PR have been shown (Guichon-Mantel *et al.* 1991). The nuclear residency of the receptor reflects a dynamic situation: the receptor diffusing into the cytoplasm and being constantly and actively transported into the nucleus (Guichon-Mantel *et al.* 1991). In the case of the spinal cord, further studies are need to unveil the functional consequences of the cytoplasmic form of PR.

Interesting data were obtained following the immunohistochemical analysis of PR. Thus, the pronounced reduction after spinal cord transection could be explained by the decrease in the level of PR mRNA. After transection, PROG had no significant effect on PR gene expression. For 25-Dx, there was a difference between the effects observed after transection, on the mRNA and protein levels. Indeed, there was no change in mRNA levels after transection while there was a decrease in the number of 25-Dx expressing cells. This discrepancy could be explained by the effects of injury on (i) protein stability; (ii) maturation, subcellular targeting processes of the protein; and (iii) the general state of the cells and membranes. PROG treatment of the transected animals produced a nice restoration of the 25-Dx labeling. Indeed, there were more 25-Dx labeled cells in the TRX + PROG group than the TRX group. The effect of PROG can be explained by its effects on maturation and targeting of the 25-Dx protein or a direct effect on the stabilization of the membranes.

Together, these observations strongly suggest different mechanisms of PROG signaling in the intact and lesioned spinal cord and point to a new potential role of the PROG membrane binding protein 25-Dx during regeneration and disease. In fact, several neuronal markers such as the choline acetyltransferase, the α3 and β1 subunits of the neuronal Na,K-ATPase and the growth-associated protein GAP-43 become sensitive to PROG after spinal cord TRX (Labombarda *et al.* 2002) and during neurodegenerative diseases (Gonzalez Deniselle *et al.* 2002; Gonzalez Deniselle *et al.* 2003). The present results suggested that regulation of these markers by PROG may involve 25-Dx, which was up-regulated by the hormone after injury, whereas PR expression was reduced in response to lesion. Clearly, the expression and significance of 25-Dx deserves to be

investigated in other models of injury and diseases of the nervous system.

The intracellular signaling cascades and the genes regulated by PROG binding to the membrane protein 25-Dx still remain unexplored. The description of closely related proteins may provide some clues. Thus, based on the sequence homology between 25-Dx and the cytokine/growth hormone/prolactin receptor family, it can be speculated that the Jak/STAT pathway may be implicated in 25-Dx function (Darnell *et al*. 1994; Selmin *et al*. 1996). 25-Dx also shares amino acid sequence homology with a protein specific to the inner zones of the rat adrenal cortex and involved in steroid hydroxylation, named inner zone antigen (IZAg) (Raza *et al*. 2001). In addition, the sequence of a novel ventral midline antigen (VEMA) cloned from the rat CNS shows strong homology with 25-Dx. The restricted distribution of VEMA, as well as several characteristics of its primary structure, suggests a role of this protein in axon guidance in the mammalian CNS (Runko *et al*. 1999). Likewise, 25-Dx may provide some guidance to regenerating axons after spinal cord injury.

Besides these hypothetical functions, it is also conceivable that PROG binding to 25-Dx directly affects characteristics of the neuronal membrane, in this way protecting nerve cells against injury-induced lipid peroxidation and excitotoxicity. Recent experiments using a model of spinal cord neurodegeneration suggest that antioxidant effects of PROG also may play a role in neuroprotection (Gonzalez Deniselle *et al*. 2002; Gonzalez Deniselle *et al*. 2003).

Immunolocalization of 25-Dx showed that its expression was restricted to the dorsal horn and central canal neurons, suggesting a potential role in pain mechanisms and restoration of sympathetic activity and mono/polysynaptic excitation of ventral horn neurons (Gootman and Cohen 1981; Furst 1999). Along this line, Ren *et al*. (2000) demonstrated an inhibitory role of PROG in the development of persistent pain and hyperalgesia at the level of the spinal cord, although the mechanisms involved in this effect were not elucidated. The question remains, however, how to postulate a role for 25-Dx in PROG neuroprotection when it was present in small neurons but not motoneurons. However, it is known that most supraspinal descending pathways are not monosynaptic, i.e. do not converge directly on motoneurons, but innervate primarily intermediate gray and dorsal horn neurons (Nacimiento *et al*. 1995) in which 25-Dx immunoreactivity was located. Therefore, part of the PROG neuroprotective effect upon motoneurons may involve the 25-Dx protein expressed in neurons of the dorsal horn and intermediate gray projecting to the ventral horn. Additionally, there may be direct effects of PROG on motoneurons mediated by their own PR (Labombarda *et al*. 2000b).

In summary, our study demonstrated the presence of the classical intracellular PR and of the new putative membrane PROG receptor 25-Dx in the rat spinal cord. These proteins diverged in their response to injury, hormone treatment and cellular localization. Their respective involvement after injury and under pathological conditions deserves further investigations, which would bring new insights into the role of PROG in neuroprotection and neurodegeneration. It is indeed very likely that PROG neuroprotection is more pleiotropic than has been previously considered, and new studies are needed to unveil the mechanisms involved.

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