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# Cellular Basis for Progesterone Neuroprotection in the Injured Spinal Cord

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## ABSTRACT

**Progesterone (PROG) exerts beneficial and neuroprotective effects in the injured central and peripheral nervous system. In the present work, we examine PROG effects on three measures of neuronal function under negative regulation (choline acetyltransferase [ChAT] and Na,K-ATPase) or stimulated (growth-associated protein [GAP-43]) after acute spinal cord transection injury in rats. As expected, spinal cord injury reduced ChAT immunostaining intensity of ventral horn neurons. A 3-day course of intensive PROG treatment of transected rats restored ChAT immunoreactivity, as assessed by frequency histograms that recorded shifts from predominantly light neuronal staining to medium, dark or intense staining typical of control rats. Transection also reduced the expression of the mRNA for the $\alpha 3$ catalytic and $\beta 1$ regulatory subunits of neuronal Na,K-ATPase, whereas PROG treatment restored both subunit mRNA to normal levels. Additionally, the upregulation observed for GAP-43 mRNA in ventral horn neurons in spinal cord–transected rats, was further enhanced by PROG administration. In no case did PROG modify ChAT immunoreactivity, Na,K-ATPase subunit mRNA or GAP-43 mRNA in control, sham-operated rats. Further, the PROG-mediated effects on these three markers were observed in large, presumably Lamina IX motoneurons, as well as in smaller neurons measuring approximately $<500\ \mu^2$. Overall, the stimulatory effects of PROG on ChAT appears to replenish acetylcholine, with its stimulatory effects on Na,K-ATPase seems capable of restoring membrane potential, ion transport and nutrient uptake. PROG effects on GAP-43 also appear to accelerate reparative responses to injury. As the cellular basis for PROG neuroprotection becomes better understood it may prove of therapeutic benefit to spinal cord injury patients.**

**Key words:** choline acetyltransferase; GAP-43; Na,K-ATPase; neuroprotection; progesterone; spinal cord injury

## INTRODUCTION

STEROID HORMONES exert beneficial and neuroprotective effects in the injured central nervous system. Within this context, it is known that early treatment with glucocorticoids accelerates recovery from spinal cord injury in humans and experimental animals (Chen et al., 1996; De Nicola, 1993; Gonzalez et al., 1996,1999; Hall,

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1992, 1993; Oudega et al., 1999;). Mechanisms of glucocorticoid action in the spinal cord include antiinflammatory and antioxidant effects, neuronal enzyme induction, and reduction of axonal die-back (Bartholdi and Schwaab, 1995; Gonzalez et al., 1996,1999; Hall et al., 1993; Oudega et al., 1999). Some of these effects may be mediated by a glucocorticoid receptor (GR; Ferrini et al., 1993; Stumpf and Duncan, 1984).

Additionally, gonadal steroids have been postulated as therapeutic agents in neuronal injury and repair (Jones, 1993). A number of reports demonstrated that progesterone (PROG) can be added to the growing list of neuroprotective steroids. In peripheral nerves, exogenous PROG or that synthesized by Schwann cells, promoted formation of new myelin sheaths after sciatic nerve lesion (Koenig et al., 1995) and stimulated the activity of the promoters of myelin genes (Desarnaud et al., 1998). Centrally, PROG effects were demonstrated in the brain and spinal cord. For example, after cortical brain contusion PROG showed antioxidant effects, reduced lipid peroxidation, facilitated cognitive recovery and prevented neuronal degeneration (Roof et al., 1994; 1997). PROG also showed therapeutic benefit in rats with middle cerebral artery occlusion (Chen et al., 1999). Gender differences in the outcome of brain injury and cerebral edema, in which females fared better than males, also pointed to a protective role of PROG (Roof and Hall, 2000; Stein and Fulop, 1998). In the spinal cord, treatment of rats with PROG increased motoneuron survival following axotomy (Yu, 1989), whereas after spinal cord injury, animals receiving PROG had a better functional and histological recovery compared to untreated injured rats (Thomas et al., 1999). Interestingly, the PROG precursor pregnenolone also facilitated recovery in rats with spinal cord injury (Guth et al., 1994). This effect may be due to increased local synthesis of PROG from pregnenolone, a step enhanced after spinal cord injury (di Michele et al., 2000). Local PROG synthesis is possible because the enzyme 3$\beta$-hydroxysteroid dehydrogenase (3$\beta$-HSD) was expressed in the spinal cord (Sanne and Krueger, 1995). In addition to this evidence, Ogata et al. (1993) reported that PROG added to cultured spinal cord neurons protected against glutamate toxicity.

In previous work, we found that PROG differentially modulated two astrocyte proteins in control and spinal cord–injured rats, namely NADPH-diaphorase (a marker of nitric oxide synthase activity) and glial fibrillary acidic protein (GFAP; Labombarda et al., 2000a). Suggestions were advanced that stimulation of reactive astrocytosis was part of a protective mechanism; in which PROG acted through a glial cell PROG receptor (PR; Labombarda et al., 2000a,b). It was recognized, however, that neuronal effects may have an important role in the biological effects of PROG in the spinal cord (Labombarda et al., 2000a; Meiri, 1986; Ogata et el., 1993; Thomas et al., 1999; Yu et al., 1989). Detection of PR immunoreactive neurons in the spinal cord also supported the possibility for neuronal effects (Labombarda et al., 2000b).

The objective of the present investigation was to analyze if some measures of neuronal function were modulated by PROG following traumatic spinal cord injury. To this end, change in choline acetyltransferase (ChAT) immunoreactivity and mRNA for growth-associated protein (GAP-43) and Na,K-ATPase subunits were examined. Functionally, ChAT is responsible for neurotransmitter synthesis, GAP-43 leads to axonal growth and regenerative responses, whereas Na,K-ATPase is needed for ion transport, cellular metabolism and maintenance of membrane potentials (Kou et al., 1995; Oestreicher et al., 1997; Stahl et al., 1986;). These parameters were selected for two reasons: First, it is known that negative regulation (ChAT- and Na,K-ATPase) or stimulation (GAP-43) of these molecules follow spinal cord injury. Second, they are regulated by glucocorticoids in the spinal cord (Curtis et al., 1993a; Gonzalez et al., 1996,1999; Gonzalez Deniselle et al., 1999). Since glucocorticoids and PROG share hormone-responsive element consensus sequences in DNA and other mechanisms of action in target tissues (Vedekis et al., 1992; Wissink et al., 1998; Zillacus et al., 1995), the possibility exists for dual hormonal regulation in the spinal cord. The results would provide new data to validate the use of PROG as a therapeutic agent for spinal cord injury, considering that PROG lacks glucocorticoid's secondary consequences (Orth and Kovacs, 1995). To our knowledge, this is the first report on the PROG regulation of neuronal function in the spinal cord after injury.

## MATERIALS AND METHODS

### *Experimental Animals*

Male Sprague-Dawley rats (250–300 g) were deeply anesthetized with Ketamine (60 mg/kg i.p.) and divided into four groups: groups 1 and 2 were sham-operated, in which case skin and muscles were cut and then sutured; in groups 3 and 4, after dorsal laminectomy, a complete spinal cord transection was performed at thoracic level T10 (Gonzalez et al., 1996,1999). In the four groups, sections were taken from the L1 segment. This model was judged suitable to evaluate neuroprotection because lesions that destroy descending tracts lead to transsynaptic degeneration of motoneurons below the lesion site (Eidelberg et al., 1989). Additionally, PROG effects on glial cells in L1 segment have already been described after transection at T10 (Labombarda et al., 2000a). Postoper-

ative care of the paraplegic rats was conducted as described by Gonzalez et al. (1996, 1999). Groups 1 and 3 received vehicle injections, whereas animals in groups 2 and 4 received PROG (Proluton, Schering Laboratories, Argentina) at the dose of 4 mg/kg at times 1 h (i.p.) and again at 24, 48, and 72 h (s.c.) postlesion. This dose of progesterone was chosen because it prevented neuronal loss after brain injury (Roof et al., 1994) and modulated glial cell parameters after spinal cord injury (Labombarda et al., 2000a). Groups 1–4 were designated control (CTL), CTL + PROG, transected (TRX), and TRX + PROG, respectively. All vehicle and PROG-treated animals were used for the different assays exactly 75 h after sham surgery or injury, and 3 h after receiving the last injection. Animal experimentation was approved by the institutional Animal Care and Use Committee. Efforts were made to reduce the number of injured animals to a minimum.

### *Choline Acetyltransferase Immunocytochemistry*

Previously published procedures that yield strong immunostaining of perikaryon and cell processes from ventral horn motoneurons (Gonzalez et al., 1999; Kou et al., 1995) were employed. Rats from groups 1–4 ($n = 6$ per group) were deeply anesthetized with ether and perfused through the heart with 0.9% NaCl followed by 4% paraformaldehyde (Gonzalez et al., 1999; Labombarda et al., 2000a). Spinal cords below the lesion site (L1 tissue level) were removed, placed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.2 for 2 h, and cryoprotected in 30% sucrose for 24 h. Spinal cord sections were embedded in Tissue Tek and placed on cryostat chuks. Fifteen micron slices were cut at −22°C and mounted on gelatine-coated glass slides. After additional washes in 0.01 M sodium phosphate buffer, 0.14 M NaCl buffer pH 7.5 (PBS) endogenous peroxidase was blocked by exposure to $H_2O_2$ during 20 min in 1% methanol. After additional washes in PBS, sections were treated with 10% goat serum for 10 min at 37°C, followed by overnight incubation in a 1/700 dilution of choline acetyltransferase (ChAT) antibody (polyclonal anti-ChAT rabbit serum, AB143, Chemicon International Inc., Temecula, CA) prepared in PBS containing 0.15% Triton ×100 and 1% goat serum. Following a PBS wash, the primary antibody was visualized with a 1/200 dilution of a biotinylated goat anti-rabbit complex (Vectastain ABC Elite Kit, Vector Labs., Burlingame, CA). The final step included 0.25 mg/mL DAB (Sigma, St. Louis, MO) for 6–8 min in the dark prepared in 0.1 M Tris, 0.01% $H_2O_2$, 2.5% nickelous ammonium sulfate pH 7.2. The sections were washed, dehydrated in graded ethanols, coverslipped with xylene and mounted with Permount. Specificity and controls for this antibody were previously described (Gonzalez et al., 1999; Kou et al., 1995).

### *Quantitative Analysis of Choline Acetyltransferase Immunoreaction and Quartile Distribution*

Staining intensity and immunoreactive cell area ($\mu m^2$) were determined for each cell by computer-assisted image analysis in Lamina IX ventral horn motoneurons. It was assumed that motoneurons larger than 500 $\mu m^2$ were $\alpha$-motoneurons and those below this size were $\gamma$-motoneurons, Renshaw cells and/or interneurons (Burke et al., 1977). The cytoarchitectonic division of Steiner and Turner (1972) was used for laminar and cell identification. The Optimas Program used in our analysis transformed differences in color intensity of the immunopositive cells into gray scale differences. The results were expressed as the inverse log of grain intensity per area (ILIGV/area; Ferrini et al., 1995).

Optical density (ILIGV/$\mu m^2$) was used to classify labeled motoneurons on a four point scale (light to very dark), following the procedure of Forger et al. (1998). Cell density scores from motoneurons of all animals in a given run were pooled, rank ordered and separated by quartile. For this division, the highest (0.56) and lowest (0.16) optical densities were considered. Motoneurons with density scores among the lowest 25% of all scores (0.16–0.26) were arbitrarily classifed as "light," whereas cells in the second, third and fourth quartiles were considered "medium" (0.26–0.36), "dark" (0.36–0.46), and "very dark" (0.46–0.56), respectively. Thus, the conversion of density reading to a nominal scale, and the subsequent analysis of the resulting relative frequency distribution with nonparametric analysis avoided the necessity of making any assumptions about the linearity of pixel density measures in our system. Data were obtained from at least six spinal cord sections per rat ($n =$ 6 animals per group).

The relative frequence distribution of intensity of motoneuron labeling were analyzed by $\chi^2$ test for independency. A significant difference in the overall $\chi^2$ was followed by partitioning analysis of contingency tables (Siegel, 1989). Differences in the mean ± SEM of the ILIGV/$\mu m^2$ (LIGV) were determined by one-way ANOVA, followed by post-hoc comparisons with the Newman-Keuls test.

### *In Situ Hybridization*

*In situ* hybridization (ISH) was carried out following previously published protocols (Gonzalez et al., 1996; Gonzalez Deniselle et al., 1999; Grillo et al., 1997). For GAP-43 ISH, a 39 mer oligonucleotide probe recogniz-

ing mouse and rat mRNA containing the sequence 5′-CGCAGCCTTATGAGCCTTATCTTCCGGCTTGA-CACCATC-3′ (Elliot et al., 1997) was used. Coding sequences for the $\alpha$3 subunit of the Na,K-ATPase subunit was 5′-CGGGAAGAGCGGCGGACAGGCTGGT-GAGCGGTGGCCGCAGA-3′ and for the $\beta$1 subunit was 5′-GCCTTCCTCCTTGGCTTTTCCGCGGGCCA-TGGTGTCTGCTCA-3′ (Gonzalez et al., 1996, Grillo et al., 1997). These oligonucleotides were synthesized by Oligos Etc, Inc. (Wilsonville, OR) and produced strong hybridization signals in ventral horn motoneurons without labeling of glial cells (Gonzalez et al., 1996; Gonzalez Deniselle et al., 1999; Grillo et al., 1997). Probes were end labeled with $^{35}$S-ATP using the enzyme terminal transferase (Boehringer-Mannheim, Germany).

For ISH spinal cords were removed and frozen at −80°C. Cryostat sections of spinal cords from groups 1–4 ($n$ = 4 animals per group) were obtained below the transection site (L1 tissue level) or a similar area taken from sham-operated rats. Sections were fixed in 2% paraformaldehyde, washed in 0.5 × SSC (1 × SSC: 0.15 M sodium chloride/0.015 M sodium citrate buffer pH 7.2) and acetylated with acetic anhydride. Hybridization proceeded overnight at 40°C with $6 \times 10^6$ cpm $^{35}$S-labeled oligo/ml in a medium consisting of 0.02% Ficoll 400, 0.02% polyvinylpirrolidone, 0.02% BSA, 50% formamide, 3 × SSC buffer, 10 mM dithiothreitol, 0.1 mg salmon sperm DNA, 1 mM EDTA, 4 $\mu$g/mL heparin, 0.4 mg/mL tRNA, and 10% dextran sulfate. At the end of hybridization, sections were washed several times in SSC, dried, dipped into Kodak NTB-2 emulsion, and exposed in the dark for 1 week (Na,K-ATPase subunits) or 2 weeks (GAP-43). Afterwards, sections were developed with Dektol (Kodak, 1:2 dilution with water), fixed, counterstained with cresyl violet, and coverslipped with Permount.

As controls for nonspecific hybridization, the signal of the $^{35}$S-labeled probe was competed with a 20-fold excess of unlabeled oligo. Under this condition, the signal was drastically reduced to background levels. Also, a specific signal was absent if the tissue was preincubated with RNAse (20 $\mu$g/mL, 30 min at 37°C) before ISH.

### *Quantitative Evaluation of In Situ Hybridization*

For the ventral horn, the number of grains per cell in neurons in which a nuclear profile could be distinguished was calculated after subtraction of the background. Quantitative grain counting was performed by computer-assisted image analysis (Bioscan Optimas II). The area of individual neurons and grain density (number of grains per unit area of soma) were measured. As already described for ChAT immunoreaction, neurons were divided into those larger than 500 $\mu$m$^2$ ($\alpha$-motoneurons) and those below 500 $\mu$m$^2$ ($\gamma$-motoneurons, Renshaw cells, interneurons). Results were expressed as the mean number of grains per mm$^2$ ± SEM. Data from about 45 cells per animal corresponding to six sections ($n$ = 4 rats per group) were combined to give an animal mean, and the animals used as independent variables. Two separate experiments carried out for each probe provided similar results. Differences in the mean ± SEM of the number of grains/mm$^2$ were determined by one-way ANOVA, followed by post-hoc comparisons with the Newman-Keults test.

## RESULTS

### *Effects of Spinal Cord Transection and Progesterone on Choline Acetyltransfersae Immunoreactivity*

As shown in Figure 1, a significant difference was found across treatment groups in the relative frequency distribution of immunoreactive intensity of ChAT-positive neurons ($\chi^2 = 237.83, p < 0.001$). In CTL rats, density distribution was similar to CTL + PROG. Density scores of the TRX group were shifted to lower (i.e., lighter) levels than those of the CTL and CTL + PROG groups ($p < 0.001$ in both cases). Whereas 96% of cells

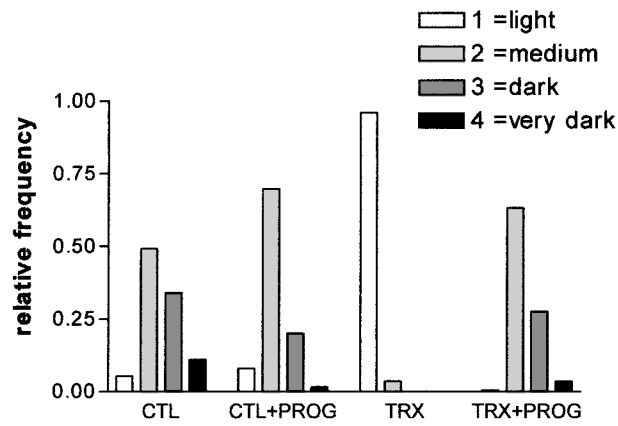


**FIG. 1.** Relative frequency distributions of the percent of neuron soma with light, medium, dark or very dark ChAT immunoreactivity (IR). Group labeling: CTL, control sham operated rats; CTL + PROG, controls receiving PROG; TRX, spinal cord transected rats; TRX + PROG, transected rats plus PROG. The distribution of label density (ILIGV/$\mu$m$^2$) was shifted to lighter values in TRX rats ($p < 0.001$ versus CTL and CTL + PROG groups) and PROG treatment of injured rats restored the distribution to CTL values (TRX + PROG versus TRX: $p < 0.001$). Statistical significance was carried out by $\chi^2$ test followed by partitioning analysis of contingency tables.

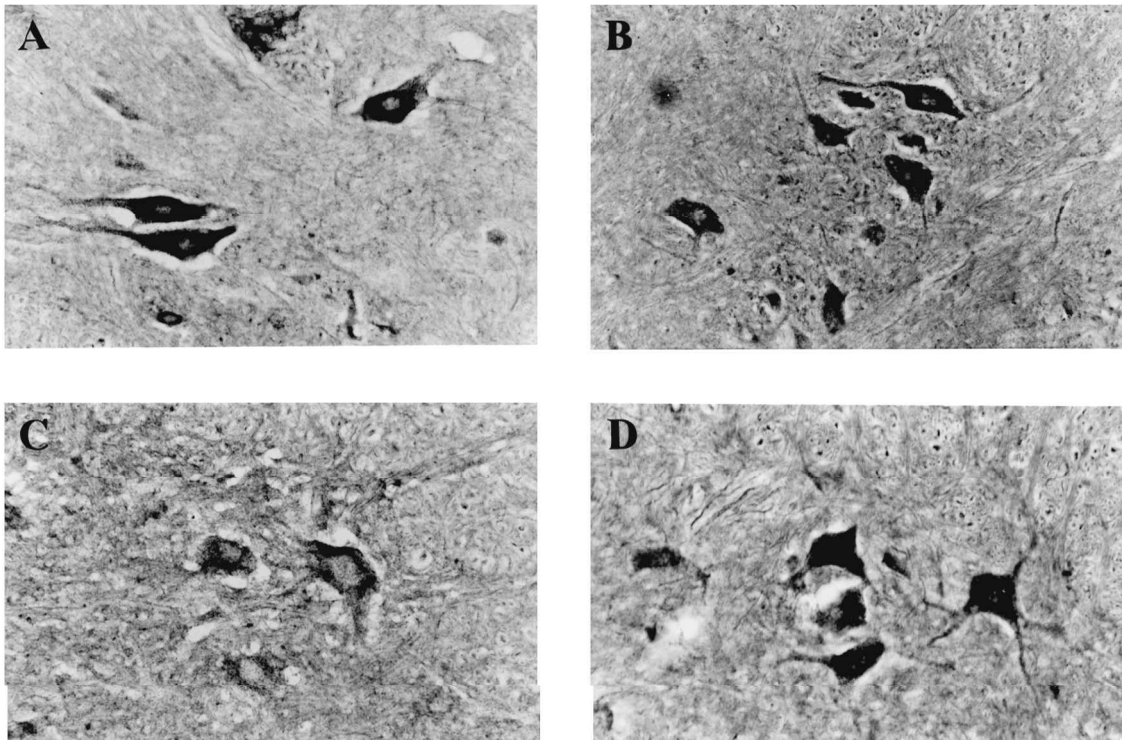


**FIG. 2.** ChAT-IR in spinal cord ventral horn neurons from CTL rats **(A),** CTL receiving PROG treatment **(B),** TRX rats **(C),** and TRX plus PROG treatment **(D)**. The intensity of ChAT-IR was reduced in TRX rats (C), whereas the intensity reverted to levels of CTL rats after PROG treatment of the lesioned group (D). Original magnification, ×400.

in the TRX group were classified as light, they amounted to about 6% in the other groups. In contrast, in the TRX + PROG group, relative neuronal frequency showed a significant shift to higher density values due to predominance of medium, dark and very dark cells ($p < 0.001$ versus TRX). Thus, neurons in the TRX + PROG group closely resembled CTL and CTL + PROG neurons.

Moreover, CTL and CTL + PROG neurons showed a strong ChAT immunoreaction intensity in perikaryon and cell processes, typical of the acetylcholine-synthesizing phenotype (Fig. 2A,B). ChAT-immunoreactivity, however, was severely reduced in TRX rats (Fig. 2C), in which these neurons presented a pale cytoplasm and few stained processes. PROG treatment of TRX rats increased ChAT immunoreactivity in perikaryon (Fig. 2D).

To study if the PROG effect was restricted to a particular neuronal population, cells showing areas of immunoreactivity larger or smaller than 500 $\mu m^2$ were separately analyzed. Data in Table 1 shows that a 50% reduction of ChAT immunoreaction intensity (ILIGV/area) was obtained in TRX rats independently of the size of neurons. Hormonal effect on this parameter was obtained equally in neurons of both sizes.

**TABLE 1. CHAT IR IN VENTRAL HORN NEURONS DIVIDED ACCORDING TO THE IMMUNOREACTIVE NEURONAL AREA IN CONTROL RATS (CTL), CTL + PROG, TRANSECTED RATS (TRX), AND TRX PLUS PROG**

| | *ChAT-IR ILIGV/$\mu m^2$* | | | |
|---|---|---|---|---|
| *Area* | *CTL* | *CTL + PROG* | *TRX* | *TRX + PROG* |
| $<500\ \mu m^2$ | $0.37 \pm 0.05^a$ | $0.32 \pm 0.03$ | $0.20 \pm 0.01^b$ | $0.34 \pm 0.04^c$ |
| $>500\ \mu m^2$ | $0.34 \pm 0.05^d$ | $0.31 \pm 0.03$ | $0.22 \pm 0.03^e$ | $0.32 \pm 0.03^f$ |

Results are the mean ± SEM of immunoreaction intensity (ILIGV/$\mu m^2$) Statistical analysis by one-way ANOVA followed by post hoc comparisons with the Newman-Keuls test, demonstrated: [a] versus [b] $p < 0.01$; [b] versus [c] $p < 0.001$; [d] versus [e] $p < 0.01$; and [e] versus [f] $p < 0.05$, respectively.

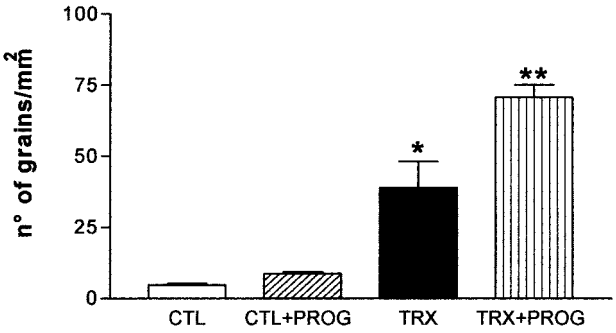


**FIG. 3.** In situ hybridization (ISH) for GAP-43 mRNA in ventral horn neurons from CTL rats (open columm), CTL receiving PROG (cross-hatched columm), TRX rats (dark columm), and TRX + ROG (vertical line columm). Group labeling as in the legend of Figure 1. Results represent the mean ± SEM of 45 cells counted per animal ($n = 4$ per group). The experiment was performed twice. Statistical analysis using ANOVA and Newman-Keuls test demonstrated a significant rise in grain density after transection (CTL versus TRX; $*p < 0.01$) and enhanced mRNA expression after PROG treatment of TRX rats (TRX versus TRX + PROG; $**p < 0.01$). PROG treatment in CTL group showed no significant effect.

## Effects of Spinal Cord Transection and Progesterone on GAP-43 mRNA

In the second phase of the study, experiments were performed to elucidate if PROG effects on ChAT immunoreactivity were accompanied by changes in GAP-43 mRNA. The reason for assessing GAP-43 mRNA rather than immunoreactivity was that rapid transport of GAP-43 out of the cell body would make quantitation of the neuronal changes more difficult to follow (Schmidt et al., 1999). Interestingly, expression of both ChAT and GAP-43 was altered in response to injury, although in opposite directions (Curtis et al., 1993b; Gonzalez et al., 1999; Johnson et al., 1995; McKinney et al., 1994; Nacimiento et al., 1996). As expected from these observations, expression of GAP-43 mRNA was virtually absent in CTL neurons (Fig. 3). In contrast, the number of grains per mm$^2$, representing probe hybridized to cytoplasmic GAP-43 mRNA was eightfold higher in TRX rats ($p < 0.01$). PROG treatment was ineffective in the CTL group, whereas a similar treatment doubled grain density in the TRX + PROG group compared to the TRX group ($p < 0.01$; Fig. 3). Table 2 shows that the effect of transection and PROG on GAP-43 mRNA was seen in neurons smaller than 500 $\mu$m$^2$ as well as in neurons larger than 500 $\mu$m$^2$. Thus, PROG had an inducing effect on GAP-43 mRNA in several neuronal populations of the ventral horn, in agreement with hormonal action on ChAT immunoreactivity. The representative photomicrographs of Figure 4 show that neurons in the CTL and CTL + PROG groups did not express GAP-43 mRNA. This molecule was moderately expressed in TRX rats and greatly stimulated in the TRX + PROG group.

### *Effects of Spinal Cord Transection and Progesterone on Na,K-ATPase Subunits mRNA*

In agreement with former data, probes coding for the $\alpha$3 and $\beta$1 subunits of the mRNA for the Na,K-ATPase labeled neurons but not glial cells (Gonzalez et al., 1996; Grillo et al., 1997). In the four groups of animals studied, that is, CTL, CTL + PROG, TRX, and TRX + PROG, grain density in neurons of all sizes ranged between 300 and 400 grains/mm$^2$ in the CTL and CTL + PROG groups (Fig. 5). In agreement with previous data in spinal cord–injured rats (Gonzalez et al, 1996), transection reduced grain density for the $\alpha$3 probe to less than 40% of CTL values ($p < 0.01$). Treatment of TRX rats with PROG reversed the effects of transection. In this case grain density of the TRX + PROG group was significantly higher than in TRX rats ($p < 0.01$; Fig. 5) and not significantly different from the CTL group. The photomicrographs of Figure 6 (A–D, dark field, magnification ×200; E–H, bright field, ×1,000) clearly show a similar, high expression level of the $\alpha$3 mRNA in the

**TABLE 2. GAP-43 mRNA EXPRESSION IN VENTRAL HORN NEURONS DIVIDED ACCORDING TO THE CELL SIZE IN CTL, CTL + PROG, TRX, AND TRX + PROG**

| | *GAP-43 mRNA expression (grains/$\mu$m$^2$)* | | | |
|---|---|---|---|---|
| *Area ($\mu$m$^2$)* | *CTL* | *CTL + PROG* | *TRX* | *TRX + PROG* |
| <500 $\mu$m$^2$ | 4.67 ± 1.92[a] | 11.77 ± 3.09 | 41.50 ± 24.64[b] | 86.5 ± 10.21[c] |
| >500 $\mu$m$^2$ | 5.50 ± 1.72[d] | 7.72 ± 2.47 | 37.25 ± 9.80[e] | 59.75 ± 11.71[f] |

Results are the mean ± SEM of number of grains/mm$^2$. Statistical analysis by one-way ANOVA followed by post hoc comparisons with the Newman-Keuls test, demonstrated: [a] versus [b] $p < 0.01$; [b] versus [c] $p < 0.001$; [d] versus [e] $p < 0.01$; and [e] versus [f] $p < 0.01$, respectively.

Group labeling as in the legend of Figure 1.

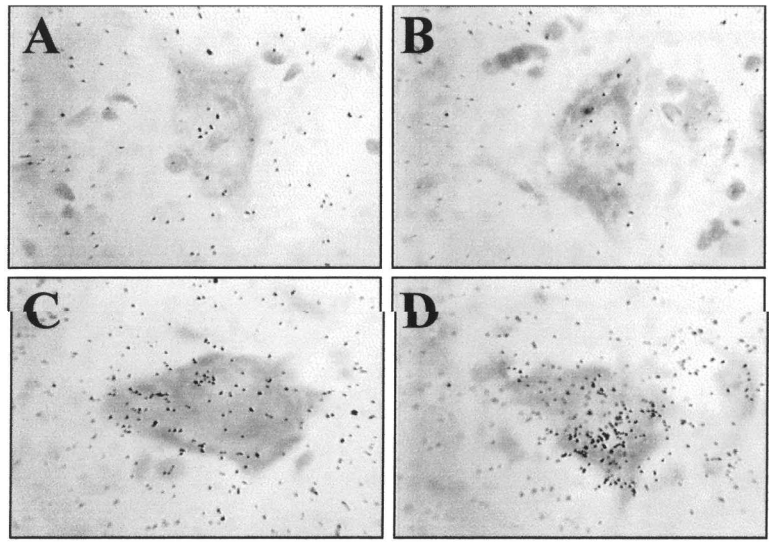


**FIG. 4.** Bright-field photomicrographs of GAP-43 mRNA in ventral horn motoneurons from CTL rats **(A),** CTL receiving PROG **(B)**, TRX rats **(C),** and TRX rats plus PROG **(D)**. The number of grains/mm$^2$ was increased after transection (C). In turn PROG treatment of the lesioned group caused a further rise in grain density (D). Original magnification, ×1000.

CTL, CTL + PROG, and TRX + PROG groups, in contrast to the low grain density of the TRX group. The low-power dark field photograph further illustrates that PROG effects in the TRX group occurred in large as well as in small size neurons.

A situation similar to that obtained with the $\alpha$3 probe was found when sections from the four experimental groups were hybridized with the $\beta$1 probe (Fig. 7). In the TRX group, average grain density was drastically reduced to less than 25% respect of CTL and CTL + PROG groups ($p < 0.001$). This reduction was totally reversed when TRX rats received PROG (Fig. 7; TRX versus TRX + PROG: $p < 0.001$). Figure 8 shows the opposite effects of spinal cord transection and PROG on Na, K-ATPase $\beta$1 subunit mRNA at the microscopic level.

To elucidate if PROG induction of higher grain density in TRX rats was confined to a single cell population (i.e., $\alpha$-motoneurons) or was also extensive to smaller size neurons, grain counting was carried out separately for cells measuring <500 or >500 $\mu$m$^2$. Table 3 indicates that while transection reduced the $\alpha$3 and $\beta$1 Na,K-ATPase mRNAs in large and small neurons, PROG treatment similarly up-regulated the messengers in cells of both sizes. These data showed that PROG effects on ChAT immunoreactivity, GAP-43 mRNA, and Na, K-ATPase subunits mRNA occurred in more than one neuronal type of the ventral horn.

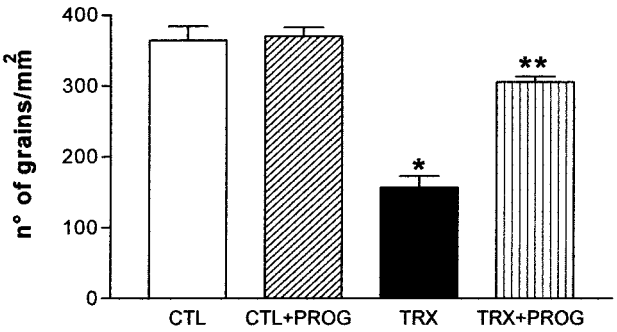


**FIG. 5.** Results of ISH for Na,K-ATPase 3 subunit mRNA in ventral horn neurons from CTL (open columm), CTL receiving PROG (cross-hatched columm), TRX (dark columm) and TRX + PROG rats (vertical line columm). Group labeling as in the legend to Figure 1. Results represent the mean ± SEM of 45 cells counted per animal ($n = 4$ per group). The experiment was performed twice. Statistical analysis using ANOVA and Newman-Keuls test demonstrated a decrease in grain density after transection (CTL versus TRX; $*p < 0.01$) and enhanced mRNA expression after PROG treatment of TRX rats (TRX versus TRX + PROG; $**p < 0.01$). PROG treatment in the CTL group showed no significant effect.

## DISCUSSION

The present investigation described the effects of PROG on three markers of neuronal function, the expression of which changed in response to spinal cord injury. Spinal cord transection and PROG treatment restored ChAT immunoreactivity and the mRNA for $\alpha$3 and $\beta$1 Na,K-ATPase subunits. PROG also enhanced the lesion-stimulated GAP-43 mRNA expression.

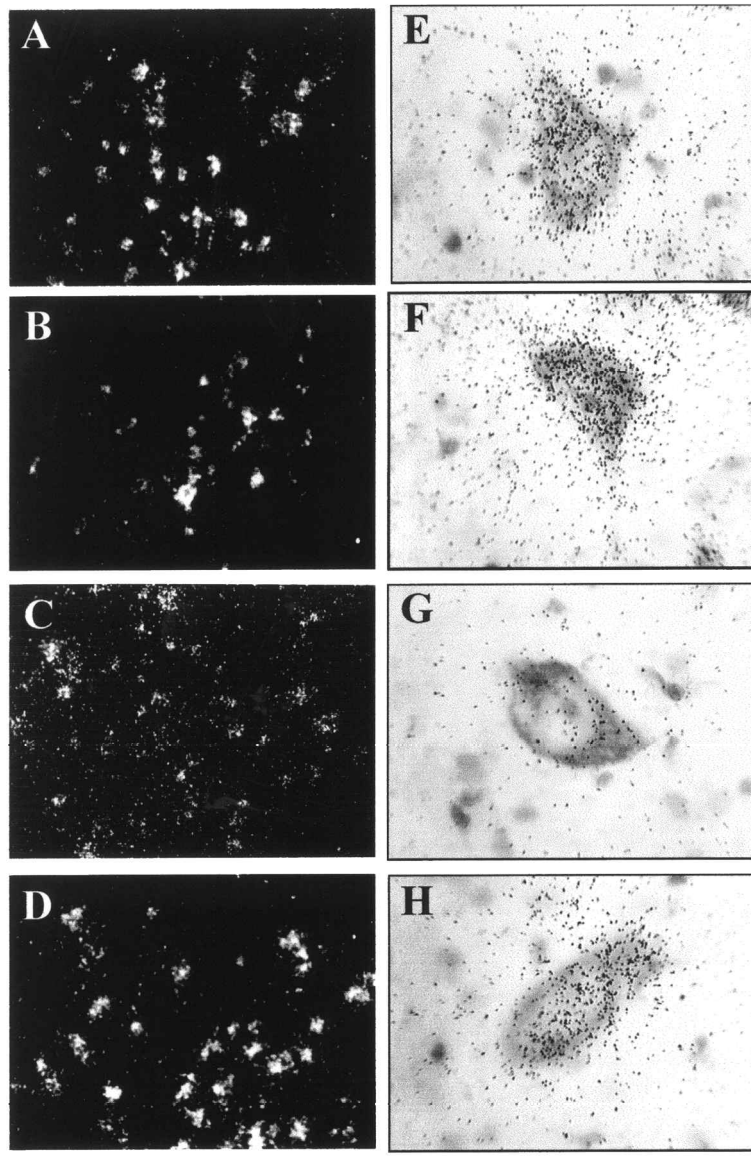


**FIG. 6.** Dark-field (left panels) and bright-field (right panel) photomicrographs representing ISH for Na,K-ATPase $\alpha 3$ subunit mRNA in ventral horn neurons. Group labeling as in the legend of Figure 1. Photomicrographs clearly show a high expression of $\alpha 3$ mRNA in the CTL **(A,E),** CTL + PROG **(B,F),** and TRX + PROG groups **(D,H),** in contrast to the low grain density of the TRX group **(C,G).** Original magnification, ×200 (A–D), ×1,000 (E–H).

The first observation made in this study was the pronounced reduction in ChAT immunostaining intensity in neuronal perikaryon from TRX rats and its reversal by PROG treatment. In contrast, ChAT was not modulated by PROG in CTL rats, indicating that at the doses given the enzyme was insensitive to PROG regulation in normal neurons. The observed PROG effects on ChAT occurred in large and small neurons, suggesting a common effect in populations expressing the cholinergic phenotype. In the case of $\alpha$-motoneurons, increases in ChAT would assure a replenishment of acetylcholine, the release of which at the neuromuscular junction is facilitated by PROG (Meiri, 1986).

In order to explain the effects of injury on ChAT expression, it has been suggested that disconnection from supraspinal and propriospinal input decreases spinal cord ChAT and acetylcholinesterase (Nacimiento et al., 1996). ChAT immunostaining reduction after injury also resembled findings in axotomized spinal cord motoneurons, in which growth factor deprivation may play a role (Rende et al., 1995; Yan et al., 1994). Regarding the molecular basis for the PROG reversal of injury effects on

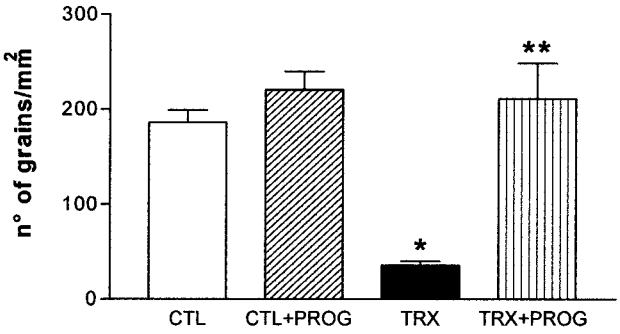


**FIG. 7.** Results of ISH for Na,K-ATPase $\beta 1$ subunit mRNA in ventral horn neurons from CTL (open columm), CTL receiving PROG (cross-hatched columm), TRX (dark columm) and TRX + PROG rats (vertical line columm). Group labeling as in the legend of Figure 1. Statistical analysis using ANOVA and Newman-Keuls test demonstrated a decrease in grain density after transection (CTL versus TRX; $*p < 0.001$) and enhanced mRNA expression after PROG treatment of TRX rats (TRX versus TRX + PROG; $**p < 0.001$). PROG treatment in the CTL group showed no significant effect.

ChAT expression, detection of PR in spinal cord neurons (Labombarda et al., 2000b) supports the possibility that PROG was acting through a classical receptor-mediated mechanism on the ChAT gene. The presence of a glucocorticoid responsive element consensus sequence in the ChAT gene, which is identical to the PROG response element (Vedekis, 1992; Zillacus et al., 1995) suggests that genomic effects could be set in motion. However, taking into consideration the above mentioned observations of Rende et al. (1995) and Yan et al. (1994), it is also possible that PROG treatment could recover ChAT indirectly by convergence with growth factors, since progestin effects on growth factors and their receptors were shown in reproductive tissues (Elizalde et al., 1998; Lange et al., 1998; Peluso et al., 1999).

In agreement with previous work, the present ISH studies confirmed that spinal cord injury reduced the expression of the mRNA for the catalytic ($\alpha 3$) and regulatory ($\beta 1$) subunits of the Na,K-ATPase (Gonzalez et al., 1996). Furthermore, we also demonstrated that intensive PROG treatment of rats with acute spinal cord injury, restored the mRNA of both subunits ot this enzyme. The hormone was without effect in CTL rats, as shown by studying ChAT immunoreactivity, suggesting that factors produced and/ or released after injury conditioned the PROG effect on these neuronal measures (Labombarda et al., 2000a; Gonzalez et al., 1996, 1999). After analyzing mRNA expression in cells of different sizes, we also concluded that PROG effects were produced not only in large size neurons of Lamina IX which may correspond to $\alpha$-motoneurons, but additionally in neurons of smaller size. It is possible, as was the case with glucocorticoids, that PROG was acting genomically to influence Na, K-ATPase subunit biosynthesis. A GR consensus sequence responsive element was reported in the Na,K-ATPase $\alpha$ and $\beta$ genes (Derfoul et al., 1998; Farman et al.,

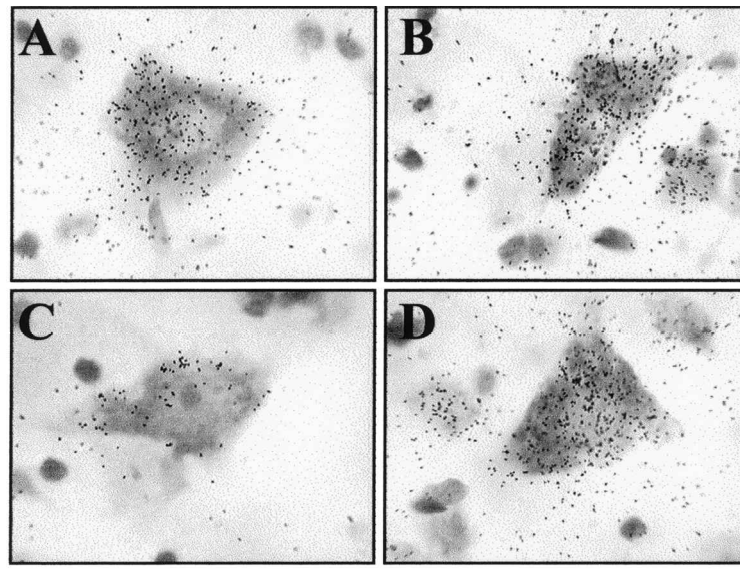


**FIG. 8.** Bright-field photomicrographs of Na,K-ATPase $\beta 1$ subunit mRNA showing ventral horn motoneurons from CTL rats **(A),** CTL receiving PROG **(B),** TRX rats **(C),** and TRX + PROG **(D).** The number of grains/mm$^2$ was decreased after transection (C). In turn, PROG treatment of the lesioned group caused a rise in grain density (D). Original magnification, $\times 1{,}000$.

**Table 3. Effects of Spinal Cord Transection and PROG on Na, K-ATPase Subunits mRNA Expression in Ventral Horn Neurons Divided According to the Cell Size in CTL, CTL + PROG, TRX, and TRX + PROG**

| | *Neurons <500 μm²* | | *Neurons >500 μm²* | |
|---|---|---|---|---|
| | *α3 subunit* | *β1 subunit* | *α3 subunit* | *β1 subunit* |
| CTL | 449.75 ±48.31 | 221.51 ± 4.33 | 390.67 ± 35.42 | 165.75 ± 20.56 |
| CTL + PROG | 475.48 ± 41.78 | 274.89 ± 29.02 | 347.16 ± 18.22 | 188.32 ± 13.94 |
| TRX | 224.14 ± 29.65 | 55.50 ± 12.30 | 142.79 ± 24.47 | 31.75 ± 3.22 |
| TRX + PROG | 425.19 ± 11.11 | 255.45 ± 32.22 | 259.00 ± 15.49 | 194.25 ± 38.00 |

Results are the mean ± SEM of number of grains/mm². Statistical analysis by one-way ANOVA followed by post hoc comparisons with the Newman-Keuls test, demonstrated that for both subunits (α3 and β1) grain density was significantly reduced in TRX rats respect of CTL, CTL + PROG, and TRX + PROG groups ($p < 0.01$). There were no significant differences between CTL, CTL + PROG, and TRX + PROG group.

Group labeling as in the legend of Figure 1.

1994). Reportedly, this DNA sequence is common to both GR and PR (Vedekis et al., 1992; Zilliacus et al., 1995).

Besides being regulated by adrenal steroids in the brain and spinal cord (Gonzalez et al., 1996; Grillo et al., 1997;), the present results suggest that the Na,K-ATPase is a target of PROG in the injured tissue. The enzyme subunits form heterodimers which are needed for the correct assembly of the catalytic subunit into the cell membrane (Watts et al., 1991). Increased expression of the mRNA of both subunits by PROG may attenuate the devastating consequences of spinal cord injury in a number of ways, because important functions including neuronal excitability, neurotransmission and nutrient uptake are subserved by the Na,K-ATPase beyond its ion transport properties (Lees, 1991; Oestreicher et al., 1997; Stahl, 1986). Jointly, these reports suggest that decrease of the Na,K-ATPase mRNA, as occurred in TRX rats, could lead to severe neuropathology (Lees, 1991). Upregulation of Na,K-ATPase subunits mRNA by PROG, as also found early using glucocorticoid treatment (Gonzalez et al., 1996) may be another important mechanism to afford hormonal neuroprotection and an attempt to restore neurotransmission after spinal cord injury.

In contrast to the opposite effects of spinal cord injury and PROG on ChAT and Na,K-ATPase subunits mRNA, both transection and PROG treatment regulated in a positive way the mRNA for GAP-43. This phosphoprotein is preferentially expressed during embryogenesis and in certain regions of the adult CNS but not by mature spinal cord motoneurons (Benowitz and Rounttenberg, 1997; Curtis et al., 1993a,b; Gonzalez Deniselle et al., 1999). Curtis et al. (1993b) demonstrated that upregulation of this molecule occurred in neurons and growing axons after compression injury of the rat spinal cord. Since the GAP-43 gene lacks a hormone responsive element recognizing the activated GR (Chao et al., 1992)—and possible PR—indirect mechanisms may be involved in the enhancement of GAP-43 mRNA due to PROG. Paracrine or autocrine effects of PROG such as modulation of growth factors in lesioned cells, may underlie the up-regulation of GAP-43 mRNA (Perrone-Bizzozero et al., 1993). Again, as observed with hormone action on ChAT immunoreactivity and Na,K-ATPase subunits mRNA, enhancement of GAP-43 message expression was obtained in neurons of different size populating the ventral horn, implying a common mechanism of PROG action in motoneurons and other neuronal types. Overall, the effects of PROG on GAP-43 could accelerate reparative responses to injury, since a link exists among GAP-43 expression, axonal sprouting, and regeneration in a lesioned tissue (Oestreicher et al., 1997).

Altogether, the results of the present work indicate that administration of PROG to rats with spinal cord injury regulated some aspects of neuronal function, in addition to its effects on glial cell proteins as described earlier (Labombarda et al., 2000a). Our work adds to existing data showing neurotrophic and neuroprotective effects of PROG after sciatic nerve lesion (Koenig et al., 1995), spinal cord compression injury (Thomas et al., 1999), axotomy (Yu, 1989), neuronal glutamate toxicity (Ogata et al., 1993), brain contusion injury and edema (Roof et al., 1994; Stein and Fulop, 1998), stroke (Roof and Hall, 2000) and ischemia (Chen et al., 1999). Thus, neurosteroids formed within the nervous tissue, or those exogenously administered may play an important role during neuronal regeneration. Future studies will unravel whether PROG will become therapeutically useful to avoid neuronal degeneration and accelerate reparative responses in the injured nervous system.

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