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PROGESTERONE UP-REGULATES NEURONAL BRAIN-DERIVED NEUROTROPHIC FACTOR EXPRESSION IN THE INJURED SPINAL CORD

S. L. GONZÁLEZ,[a,b] F. LABOMBARDA,[a,b] M. C. GONZÁLEZ DENISELLE,[a,b] R. GUENNOUN,[c] M. SCHUMACHER[c] AND A. F. DE NICOLA[a,b]*

[a]*Laboratory of Neuroendocrine Biochemistry, Instituto de Biología y Medicina Experimental, Obligado 2490, 1428 Buenos Aires, Argentina*

[b]*Department of Biochemistry, Faculty of Medicine, University of Buenos Aires, Buenos Aires, Argentina*

[c]*INSERM U488, Hôpital de Bicêtre, 80 rue du Général Leclerc, 94276 Kremlim-Bicêtre, Paris, France*

Abstract—Progesterone (PROG) provides neuroprotection to the injured central and peripheral nervous system. These effects may be due to regulation of myelin synthesis in glial cells and also to direct actions on neuronal function. Recent studies point to neurotrophins as possible mediators of hormone action. Here, we show that the expression of brain-derived neurotrophic factor (BDNF) at both the mRNA and protein levels was increased by PROG treatment in ventral horn motoneurons from rats with spinal cord injury (SCI). Semiquantitative *in situ* hybridization revealed that SCI reduced BDNF mRNA levels by 50% in spinal motoneurons (control: 53.5±7.5 grains/mm^2 vs. SCI: 27.5±1.2, $P<0.05$), while PROG administration to injured rats (4 mg/kg/day during 3 days, s.c.) elicited a three-fold increase in grain density (SCI+PROG: 77.8±8.3 grains/mm^2, $P<0.001$ vs. SCI). In addition, PROG enhanced BDNF immunoreactivity in motoneurons of the lesioned spinal cord. Analysis of the frequency distribution of immunoreactive densities (χ^2: 812.73, $P<0.0001$) showed that 70% of SCI+PROG motoneurons scored as dark stained whereas only 6% of neurons in the SCI group belonged to this density score category ($P<0.001$). PROG also prevented the lesion-induced chromatolytic degeneration of spinal cord motoneurons as determined by Nissl staining. In the normal intact spinal cord, PROG significantly increased BDNF inmunoreactivity in ventral horn neurons, without changes in mRNA levels. Our findings suggest that PROG enhancement of endogenous neuronal BDNF could provide a trophic environment within the lesioned spinal cord and might be part of the PROG activated-pathways to provide neuroprotection.

Key words: steroid hormones, neurotrophins, neuroprotection, *in situ* hybridization, immunohistochemistry.

*Correspondence to: A. F. De Nicola, Laboratory of Neuroendocrine Biochemistry, Instituto de Biología y Medicina Experimental, Obligado 2490, 1428 Buenos Aires, Argentina. Tel: +54-11-4783-2869; fax: +54-11-4786-2564.
E-mail address: denicola@dna.uba.ar (A. F. De Nicola).

Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; CTL, control; ILIGV/area, inverse logarithm of grain intensity per area; ISH, *in situ* hybridization; PBS, phosphate-buffered saline; PNS, peripheral nervous system; PR, progesterone receptor; PROG, progesterone; SCC, sodium citrate/sodium chloride buffer; SCI, spinal cord injury.

Steroid hormones produce trophic and neuroprotective effects both in the CNS and peripheral nervous system (PNS). In this respect, several studies have been carried out to elucidate the cellular and molecular pathways involved in glucocorticoid effects in spinal cord injury (SCI; Hall, 1992, 1993; De Nicola, 1993; Chen et al., 1996; Oudega et al., 1999), androgen effects on motoneuron survival (Jones et al., 2001) and estradiol protection in cell and animal models of injury and neurodegeneration (Behl, 2002; García-Segura et al., 2001). A growing list of publications also gives evidence of the protective and trophic effects of progesterone (PROG). In the PNS, PROG promotes myelination (Koenig et al., 1995; Azcoitia et al., 2003; Desarnaud et al., 1998; Magnaghi et al., 2001) and this stimulatory effect can be extended to the CNS. Indeed, PROG stimulates myelination in organotypic slices cultures of 7-days-old (P7) rat and mouse cerebellum (Ghoumari et al. 2003) and partially reverses toxin-induced demyelination in old male rats (Ibanez et al., 2003). PROG also facilitates cognitive recovery and prevents neurodegeneration after cortical contusion (Roof et al., 1994, 1997; Stein and Fulop, 1998; Stein, 2001). Gender differences in the outcome of brain injury and cerebral edema also pointed to a protective role of PROG (Stein and Fulop, 1998; Roof and Hall, 2000). PROG increases motoneuronal survival following axotomy (Yu, 1989), and after spinal contusion, animals receiving PROG have a better functional and histological outcome compared with untreated injured rats (Thomas et al., 1999).

In previous work, we have shown that PROG regulates some key features of neuronal function after SCI (Labombarda et al., 2002) and in a mouse model of neurodegeneration (González Deniselle et al., 2002, 2003). In spinal motoneurons, PROG restores both injury-decreased choline acetyltransferase immunoreactivity and mRNA expression for the neuronal Na,K-ATPase and further increased GAP-43 mRNA levels (Labombarda et al., 2002). PROG also increases immunostaining for myelin-basic protein and the number of NG2+ progenitors after SCI (De Nicola et al., 2003). The localization of both an estrogen-inducible (Monks et al., 2001) and estrogen-insensitive PROG receptor (PR) in neurons and glial cells of the rat spinal cord (Labombarda et al., 2000b, 2003) implicates the involvement of the classical PR in neuroprotection. However, the presence of a membrane receptor for PROG in the spinal cord (Labombarda et al., 2003) and PROG metabolism to reduced derivatives (Guennoun et al., 2001) which modulate the activity of neurotransmitter receptors

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(Majewska et al., 1986; Rupprecht et al., 1996) suggests multiplicity of mechanisms in PROG action.

Brain-derived neurotrophic factor (BDNF), a member of the nerve growth factor family of trophic factors, mimics some of these PROG effects on the spinal cord. For example, application of BDNF prevents the axotomy-induced decrease of choline acetyl transferase in motoneurons (Yan et al., 1994), stimulates sprouting of cholinergic fibers and hindlimb stepping (Jakeman et al., 1998; Ankeny et al., 2001) and increases the expression of the regeneration-associated gene GAP-43 after SCI (Kobayashi et al., 1997). Additionally, BDNF administration decreases edema formation (Winkler et al., 2000) and promotes the recovery of myelin-basic protein after compression-induced SCI (Ikeda et al., 2002). Neurotrophic factors and their receptors are present not only in developing but also in adult spinal cord neurons (Dreyfus et al., 1999; Schober et al., 1999; Buck et al., 2000), indicating they may play an important role for neuronal survival (Thoenen, 1995) and axonal regeneration (Thoenen, 1995; Sayer et al., 2002).

Recent data support that steroid hormones interplay with neurotrophins in the CNS (Forger et al., 1998; Ianova et al., 2001; Solum and Handa, 2002). Motoneurons of the spinal cord express PR (Labombarda et al., 2000b) and, as already stated, neurotrophins and their cognate receptors (Schober et al., 1999). Although colocalization studies are lacking, this cellular distribution suggests that PROG modulation of motoneuron parameters may involve modulation of endogenous trophic factors. Therefore, the aim of the present study was to explore whether endogenous BDNF expression was regulated by PROG in neurons of the normal and injured spinal cord. From the therapeutic point of view, treatment of animals or patients with SCI with PROG may be advantageous, in view of the reported difficulties with delivery of trophic factors to the injured tissue (Chun et al., 2000).

EXPERIMENTAL PROCEDURES

Animals and surgical procedures

Male Sprague–Dawley rats (250–300 g) were deeply anesthetized with a mixture of ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). In sham-operated controls, soft tissues were cut and then sutured. SCI was performed under an operating microscope by transecting the spinal cord at thoracic level T10 with iridectomy scissors (González et al., 1996, 1999; Cummings and Stelzner, 1988; Labombarda et al., 2002). The completeness of the transection was verified by passing the sharp edge of a 25G needle through the lesion site. Bleeding was controlled with Gelfoam (Pharmacia & Upjohn, Kalamazoo, MI, USA) and body temperature was maintained at 37 °C. A group of rats with SCI received vehicle or four injections of PROG (Schering Laboratories, Argentina; 4 mg/kg b.w.) at 1 h and again at 24, 48 and 72 h (s.c) post-lesion. This protocol was chosen because it prevented neuronal loss after brain injury in rats (Roof et al., 1994), modulated both glial and neuronal parameters after SCI (Labombarda et al., 2000a, 2002) and improved clinical and histological outcome after spinal cord contusion (Thomas et al., 1999). Four experimental groups were analyzed: 1) sham-operated untreated controls (CTL); 2) CTL with PROG treatment (CTL+PROG); 3) untreated lesioned rats (SCI) and 4) SCI with PROG treatment (SCI+PROG). As previously reported, this PROG treatment resulted in 925-fold and 50-fold higher PROG levels in the plasma and spinal cord of the SCI+PROG group as compared with SCI group (Labombarda et al., 2003).

Postoperative care included warming, twice-daily urinary bladder expression, and hand feeding until full recovery. All vehicle and progestin-treated animals in the different experimental groups were allowed to survive for 75 h following surgery, i.e. 3 h after receiving the last PROG or vehicle injection and were prepared and handled identically until kill (González et al., 1996, 1999; Labombarda et al., 2002). Studies were carried out at lumbar L1 level caudal from the lesion site. Our model was judged suitable to evaluate neuroprotection because transection at T10 interrupts supraspinal descending tracts as well as propriospinal pathways to lumbar segments, leading to transsynaptic degeneration of motoneurons below the lesion site (Eidelberg et al., 1989; Nacimiento et al., 1995). In addition, PROG effects on glial cells and neurons in the L1 segment have already been described after transection at T10 (Labombarda et al., 2000a, 2002). Animal procedures followed the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Guide, Instituto de Biología y Medicina Experimental, Assurance Certificate N A5072-01) and were approved by the Institute's Animal Care and Use Committee. Every effort was made to minimize animal suffering and to reduce the number of lesioned animals to a minimum.

In situ hybridization (ISH)

ISH was carried out under RNAse-free conditions following previously published protocols (González et al., 1996; Labombarda et al., 2002). Cryostat sections obtained from the L1 spinal level below the lesion site or a similar segment of sham-operated rats were fixed in 2% paraformaldehyde, washed in 0.5× sodium citrate/sodium chloride buffer (SCC; 1× SCC: 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0), dried and acetylated with acetic anhydride. To detect BDNF-specific mRNA, we used a 48-mer synthetic oligonucleotide probe (Oligos Etc, Inc., Wilsonville, OR, USA) containing the complementary sequence to bp 562–609 of rat BDNF (Maisonpierre et al., 1991). The probe was end labeled with (^{35}S)dATP using the enzyme terminal transferase (Boehringer-Mannheim, Germany). Hybridization was carried out using 10^6 c.p.m. of ^{35}S-labeled probe in 100 μl hybridization cocktail containing: 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% BSA, 50% formamide, 3× SCC buffer, 10 mM dithiothreitol, 0.1 mg/ml salmon sperm DNA, 1 mM EDTA, 4 μg/ml heparin, 0.4 mg/ml tRNA and 10% dextran sulfate. After overnight hybridization at 42 °C, sections were washed several times in SCC, dried, dipped into Kodak NTB-2 emulsion and exposed in the dark for 8 weeks. Sections were then developed with Dektol (Kodak; 1:2 dilution with water), fixed in Ektaflo fixer, counterstained with Cresyl Violet and coverslipped with Permount. A set of slides was hybridized as above in the presence of 20-fold excess of unlabeled probe. Under this condition, the signal was drastically reduced to background levels. In addition, we also observed that the specific signal was absent when CTL tissue sections were preincubated with RNAse (20 μg/ml, 30 min at 37 °C) before ISH.

Semiquantitative evaluation of ISH

The number of silver grains per cell representing probe hybridization to BDNF mRNA was determined over motoneurons of Lamina IX showing a clear nuclear profile. The cytoarchitectonic division of Steiner and Turner (1972) was used for laminae and cell identification. Grain counting was performed by computer-assisted image analysis (Bioscan Optimas VI) and calculated after background substraction (González et al., 1996; Labombarda et al., 2002). The area of individual neurons and grain density (number of grains per unit area of soma: grains/mm^2) were measured.

Results were expressed as the mean number of grain density±S.E.M. Data from 50 to 60 neurons per animal corresponding to six sections (n=4–5 rats per group) were combined to obtain a mean value per animal, and the animals were used as independent variables. The experiment was repeated twice with similar results. Differences in the mean±S.E.M. were determined by one-way analysis of variance (ANOVA), followed by post-hoc Newman-Keuls comparison test. Images were acquired at the same magnification using a digital camera Panasonic GP-KR222 connected to an Olympus BH2 microscope and the image analysis software Bioscan Optimas VI.

Immunocytochemistry

Animals were deeply anesthetized and perfused intracardially with phosphate-buffered saline (PBS, pH 7.4) followed by freshly prepared 4% paraformaldehyde in PBS. Spinal cord L1 segments were carefully dissected out and stored in the same fixative for 2 h (4 °C). After post-fixation, spinal cord segments were cryoprotected in 20% sucrose with the same phosphate buffer and stored overnight (4 °C). The L1 segment below the lesion site or a comparable region in sham-operated animals were embedded and frozen in Tissue-Tek (OCT compound; Miles Inc., USA). Transverse sections (15 μm) were cut in a cryostat maintained at −20 °C and placed on gelatin-coated slides. Previously used immunocytochemical procedures were followed (González et al., 1999; Labombarda et al., 2002). Briefly, for BDNF immunostaining, tissue sections were sequentially exposed to H_2O_2 in methanol (1:100) for 30 min and then to 10% goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS at 37 °C for 10 min. Sections were incubated overnight at 4 °C with a 1/500 dilution of the primary antibody raised against purified BDNF (N-20, sc: 546, polyclonal rabbit antiserum; Santa Cruz Biotechnology, Santa Cruz, CA, USA), containing 1% goat serum. Sections were then washed twice in PBS, incubated with biotinylated goat anti-rabbit complex and processed using the Vectastain ABC kit (Vector Laboratories). Development was carried out using 0.25 mg/ml diaminobenzidine tetrachloride (Sigma Chemical Co., St. Louis, MO, USA) containing 0.01% (vol/vol) H_2O_2. To obtain consistency of immunostaining, sections from all experimental groups were stained simultaneously and sections from different animals were included in the same slide. This procedure eliminated conflicts that may arise using different batches. To minimize interexperimental variability, conditions of the procedures were kept rigorously throughout the assays. The chromogenic reaction was monitored microscopically. Omission of the primary antibody or preadsorption of the primary antiserum with the blocking peptide supplied by the manufacturer blunted the immunocytochemical signal. The specificity of this antibody has been validated (Skup et al., 2002). The sections were then washed in PBS, dehydrated in graded ethanols and xylene, and mounted with Permount (Fisher Scientific, Pittsburgh, PA, USA).

Analysis of BDNF immunoreaction and quartile distribution

Staining intensity and immunoreactive cell area (μm^2) were determined for each motoneuron of lamina IX (Steiner and Turner, 1972) by computer-assisted image analysis (Labombarda et al., 2002). 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 logarithm of grain intensity per area (ILIGV/area; Ferrini et al., 1995; Labombarda et al., 2002). Sections were mounted and processed simultaneously under identical operating conditions, such as light beam, wavelength and gray-scale threshold throughout the experiment. Images were acquired at the same magnification using a digital camera Panasonic GP-KR222 connected to an Olympus BH2 microscope and the image analysis software Bioscan Optimas VI (Labombarda et al., 2002, 2003).

Optical density (ILIGV/area) measures were then 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. Motoneurons with density scores among the lowest 25% of all scores (0.08–0.13) were arbitrarily classified as “light,” whereas cells in the second, third, and fourth quartiles were classified as “medium” (0.13–0.18), “dark” (0.18–0.23), and “very dark” (0.23–0.28), respectively. Thus, the conversion of density reading to a nominal scale, and the subsequent analysis of the resulting relative frequency distributions with nonparametric statistic avoided the necessity of making any assumptions about the linearity of pixel density measures in our system (Forger et al., 1998). Data were obtained from at least six spinal cord sections per rat (n=4–5 animals per group). The relative frequency distribution of intensity was analyzed by χ^2 test for independency. A significant difference in the overall χ^2 was followed by partitioning analysis of contingency tables (Siegel and Castellan, 1989).

In addition, randomly selected areas of ventral horn neuropil were sampled from each section. After background substraction and gray-scale threshold determination, BDNF-immunopositive fiber density was quantified by image analysis (Optimas, Bioscan VI) according to previously reported protocols (Abrahám et al., 2000; Skup et al., 2002). Surface area covered by BDNF-positive fibers and the length of positive fibers were computed and expressed as BDNF-immunopositive fiber density (μm immunopositive fiber length/30 mm^2). Quantitative analysis was performed by one-way ANOVA, followed by post hoc comparisons with the Newman-Keuls test.

Light microscopy and Nissl staining

Anesthetized animals from the four experimental groups were perfused with 0.9% (w/v) cold NaCl and spinal cords were removed and dissected as specified above. Spinal segments were fixed in 10% (v/v) formalin for 48 h, dehydrated, and processed for standard paraffin embedding. Five micrometer spinal cross-sections were stained with 0.5% Cresyl Violet (Sigma), dehydrated in graded ethanols and xylene, and coverslipped with Permount. In our histological preparations, neurons were classified as normal, “mild” chromatolytic and “severe” chromatolytic, according to previously reported criteria (Young, 1966; Price and Porter, 1972; Clatterbuck et al., 1994; Nacimiento et al., 1995). Normal-basophilic neurons presented a triangular shape, mottled nucleus, dense nucleolus and a clearly defined cytoplasm containing distinct Nissl bodies. Neurons classified as mild chromatolytic displayed some degree of nuclear eccentricity, rounded shape and normal Nissl bodies with a tendency to accumulate at the periphery of the cell, as described by Nacimiento et al. (1995). Motoneurons exhibiting the classic signs of severe chromatolysis showed eccentric nuclei, rounded perikarya and Nissl material finely dispersed and present at the edge of the cell resulting in a “ghostly appearance” (Price and Porter, 1972). Double counting of neurons was unlikely because of the 50 μm spacing selected between consecutive sections (Labombarda et al., 2003). In addition, the presence of a prominent nucleolus avoided the counting of the same cell twice.

Sections were analyzed in a double blinded manner by different investigators. Analysis of the relative frequency distributions of normal and chromatolytic neurons was performed with nonparametric statistics (χ^2 test) followed by partitioning analysis of contingency tables (Siegel and Castellan, 1989).

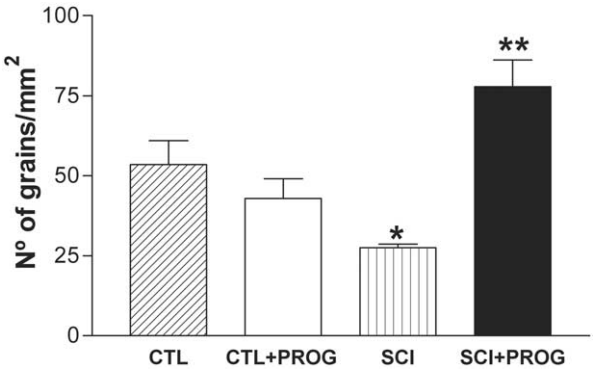

Fig. 1. Semiquantitative ISH for BDNF mRNA in ventral horn neurons from CTL rats (cross-hatched column), CTL receiving PROG (open column), rats with SCI (vertical line column) and rats with SCI receiving PROG treatment (dark column). Results represent the mean±S.E.M. of 50–60 cells counted per animal (n=4–5 per group). Statistical analysis demonstrated that grain density (number of grains/mm^2) was similar in CTL and CTL+PROG groups (P=n.s.). SCI reduced mRNA levels by 50% in large motoneurons as compared with CTL (* $P<0.05$), whereas the SCI+PROG group showed a significantly higher BDNF mRNA levels vs. untreated rats with SCI (** $P<0.001$), by Newman-Keuls test after one-way ANOVA.

RESULTS

BDNF-mRNA expression

In agreement with previous reports, BDNF mRNA was expressed within large ventral horn neurons of CTL rats (Buck et al., 2000; Dreyfus et al., 1999; Ikeda et al., 2002). In the present study, observations were restricted to large cells (>500 μm^2) of Rexed Lamina IX, considered α-motoneurons based on size and anatomical localization. Fig. 1 shows that the number of silver grains/mm^2 clustered over the neurons was similar in the CTL and CTL+PROG groups (P=n.s). Quantitative grain counting also revealed that SCI reduced BDNF mRNA levels by 50% compared with CTL values (CTL: 53.5±7.5 grains/mm^2 vs. SCI: 27.5±1.2, $P<0.05$). However, a 3 day course of PROG treatment of the injured animals elicited a three-fold increase in BDNF mRNA labeling. In this case, grain density was significantly higher in the SCI+PROG animals (77.8±8.3 grains/mm^2) than in the SCI group ($P<0.001$, Fig. 1).

The representative photomicrographs of Fig. 2 clearly show similar grain density levels of BDNF mRNA in CTL (A) and CTL+PROG (B) motoneurons, in contrast to the low grain density exhibited by neurons after SCI (C). BDNF mRNA expression was significantly enhanced by PROG treatment of injured animals (Fig. 2D).

BDNF immunocytochemistry

To support that variations in BDNF mRNA expression detected across the experimental groups were of potential functional significance, protein expression of BDNF was assessed by immunocytochemistry. Previous work demonstrated that motoneurons are immunoreactive toward BDNF (Furukawa et al., 1998; Skup et al., 2002).

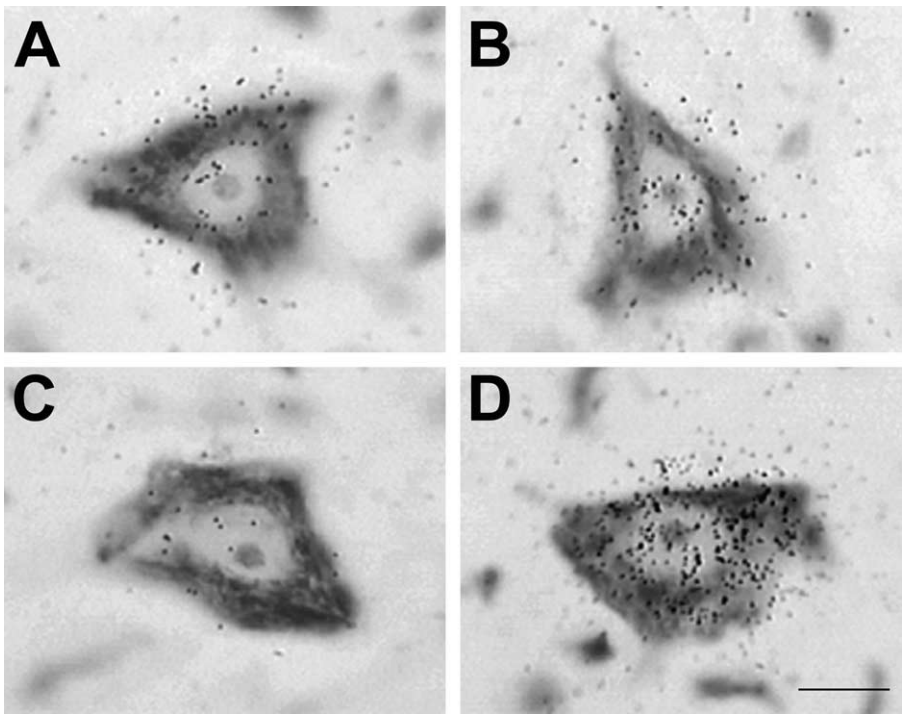

Fig. 2. Representative brightfield photomicrographs showing ISH for BDNF mRNA expression in ventral horn motoneurons from a CTL rat (A), a CTL rat receiving PROG (B), a rat with SCI (C) and a rat with SCI receiving PROG treatment (D). The number of grains was decreased after SCI (C). In turn, PROG administration to the lesioned group significantly enhanced grain density (D). This plate and Figs. 4 and 5 were generated, without alterations, from digital images. Scale bar in D=15 μm (also applies to A–C).

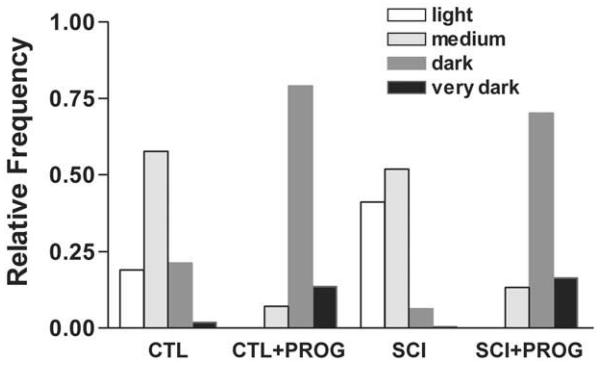

Fig. 3. Quartile distribution of BDNF immunoreactive cells in the spinal cord. Optical densities scores were used to classify labeled neurons in a four-point scale (light, medium, dark and very dark). The relative frequency distributions of BDNF immunoreactive densities were analyzed by χ^2 test for independency followed by partitioning analysis of contingency tables (χ^2=812.73, P<0.0001). Group labeling: CTL, CTL sham-operated rats; CTL+PROG, CTLs receiving PROG; SCI, animals with SCI; SCI+PROG, SCI group receiving PROG treatment. The distribution of label density (ILIGV/μm^2) was shifted to lighter values in SCI animals (P<0.001 vs all other groups) whereas PROG treatment shifted the distribution to darker values in both the CTL and SCI groups.

Fig. 3 shows that a significant difference existed across treatment groups in the frequency distribution of BDNF-immunoreactive density (χ^2=812.73, P<0.00001). PROG administration to CTL rats shifted the density distribution to higher values (i.e. dark and very dark staining) than those observed in the CTL group (P<0.05). In this case, 80% of neurons were classified as dark in the CTL+PROG group, whereas only 21% scored in this category in CTL rats. After SCI, density scores were shifted to lower values (i.e. light and medium staining) respect to all other groups (P<0.001 for each case). Whereas 40% of neurons in the SCI group scored as light, they amounted to about 20% in the CTL animals.

In contrast, the SCI+PROG group showed a significant shift to higher density values due to preponderance of medium, dark and very dark neurons. In these animals, 70% of motoneurons scored as dark whereas only 6% in the untreated SCI group belonged to this category (P<0.001). Consequently, the density profile of SCI+PROG was similar to that of intact animals receiving PROG (P=n.s.). In addition, we observed that the immunoreactive neuronal area was similar across all experimental groups (data not shown). Considering that treatment could not modify the immunoreactive area, it is possible that changes in ILGV/μm2 were elicited by an increase in the intensity of BDNF immunoreaction. The effect of PROG on BDNF immunoreactivity is further demonstrated in Fig. 4A–D. Fig. 4A and B show the increased BDNF-inmunolabeling in cell bodies of CTL+PROG as compared with CTL rats. Fig. 4C presents the severely reduced immunoreactivity of SCI motoneurons, with predominance of pale cytoplasm and few detectable stained processes. This histological profile contrasts with the SCI+PROG group, showing strong BDNF immunostaining of perikarya and fiber network (Fig. 4D).

BDNF immunoreaction density of cell processes also presented group differences. Quantitative analysis revealed that immunoreactive fiber density surrounding motoneurons of the CTL and CTL+PROG groups amounted

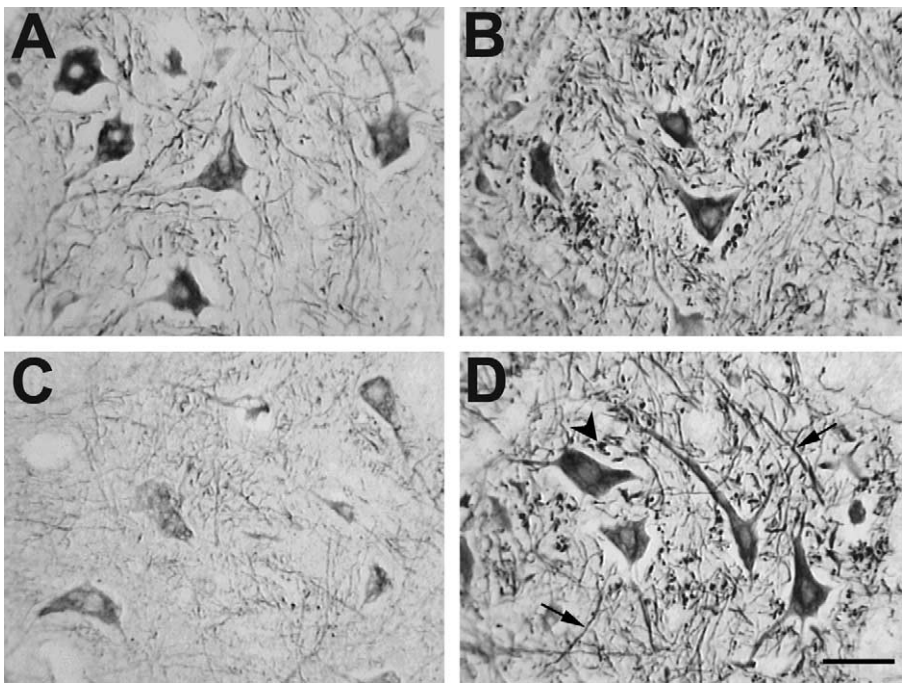

Fig. 4. Immunocytochemical localization of BDNF to spinal motoneurons from a representative CTL sham-operated rat (A), CTL rat receiving PROG (B), rat with SCI (C) and rat with SCI receiving PROG treatment (D). PROG administration to SCI rats (D) led to an enhancement of BDNF immunoreactivity in dense plexus of fibers (thin arrows) and terminal swellings (arrowheads) surrounding large neurons of Lamina IX. Note in (D) the strong BDNF immunolabeling of motoneuron somata, neuropil and fibers. Scale bar in D=50 μm (also applies to A–C).

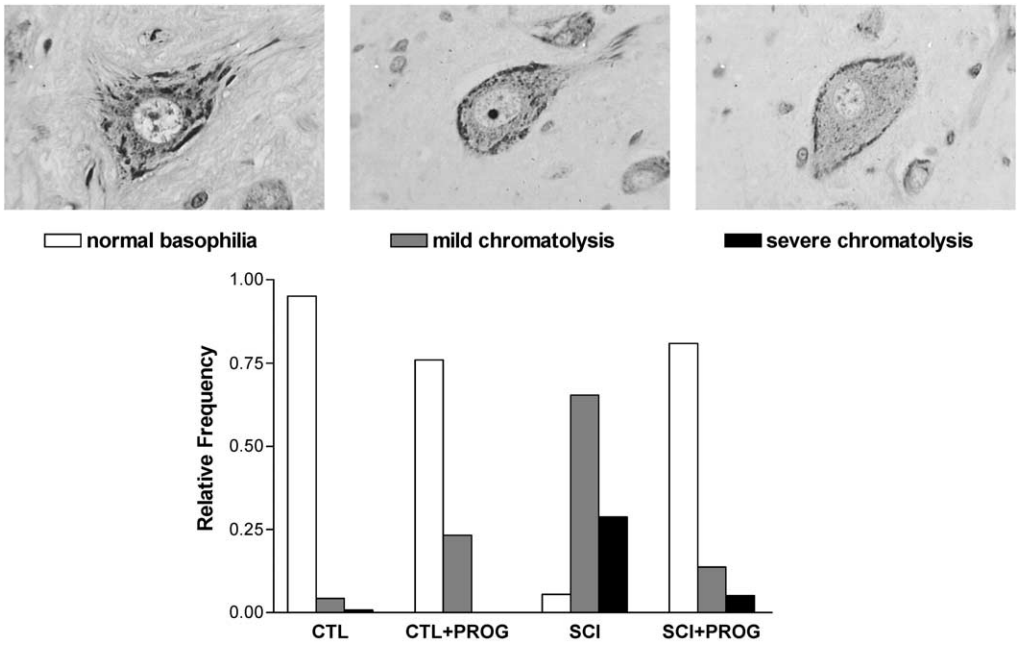

Fig. 5. Frequency histograms showing different motoneuron phenotypes in CTL, CTL+PROG, SCI and SCI+PROG groups (group labeling as in the legend to Fig. 1). In the upper portion of this figure, representative Cresyl Violet-stained motoneurons were selected to show the three point scale classification: normal basophilia (left-hand photograph), mild chromatolysis (center) or severe chromatolysis (right-hand photograph). After SCI, only 5% of motoneurons appeared normal and 30% of motoneurons correspond to the severe phenotype, showing eccentric nucleus, disappearance of Nissl bodies and peripheral accumulation of remaining Nissl bodies. In the SCI group receiving PROG, the Nissl pattern appeared normal in 81% of neurons, and only few cells showed mild chromatolytic changes.

to 5983±396 μm/30 mm^2 and 5972±523 μm/30 mm^2 respectively. After SCI (Fig. 4C), there was a dramatic loss of BDNF positive fibers (SCI: 3595±560 μm/30 mm^2, $P<0.05$ vs. CTL and CTL+PROG). whereas in SCI+PROG animals a plexus of heavily labeled neurites appeared (Fig. 4D). Statistically, the BDNF-immunopositive fiber network was significantly enhanced after PROG treatment of SCI rats (SCI+PROG: 9233±603 μm/30 mm^2, $P<0.001$ vs. SCI). In addition (Fig. 4D), numerous granular and intensive BDNF immunopositive deposits resembling terminal swellings were detected in apposition to neuronal perikarya (Skup et al., 2002).

While the focus of this study was on motoneurons, changes in BDNF immunoreactivity were also seen in other parts of the spinal cord, such as the dorsal horn. In this case, immunoreaction density was increased by PROG treatment in both CTL and SCI groups (40% increase, data not shown). Thus, the PROG effect in the dorsal horn resembled data for BDNF immunoreaction in motoneurons.

PROG regulation of chromatolysis

Since chromatolysis is a typical incident of injured neurons, we studied if PROG neuroprotection after SCI inhibited this process and preserved Nissl staining. First, we observed that motoneurons from CTL and CTL+PROG animals showed a normal basophilia and were characterized by clusters of Nissl bodies in multiple locations throughout the cytoplasm (Fig. 5, left-hand photograph). Following SCI, most motoneurons were mild chromatolytic (Fig. 5, middle photograph), or presented the severe type (Fig. 5, right-hand photograph) and few remained normal. The severe degenerating motoneurons contained granular dispersion of Nissl bodies, displacement of the nucleus to the cell membrane, rounded shape and faintly stained cytoplasm, resulting in a “ghostly appearance.” Analysis of the frequency histograms (Fig. 5) demonstrated that significantly differences existed among the experimental groups ($\chi^2=210.53$, $P<0.0001$). After SCI, only 5% neurons remained normal, and most motoneurons scored as mild (65%) or severe (30%) chromatolytic ($P<0.001$ vs. CTL). In the SCI+PROG animals, Nissl staining appeared normal in 81% ventral horn neurons, whereas just a minority showed mild (14%) or the severe type (5%) of chromatolysis ($P<0.001$ vs. SCI, Fig. 5).

Despite the fact that SCI produced chromatolysis and that PROG treatment restored the Nissl staining of injured motoneurons, cell size was not apparently modified by these procedures. Thus, morphometric analysis of Cresyl Violet-stained cells demonstrated similar areas in CTL (726±23 μm^2), CTL+PROG (781±17), SCI (793±65) and SCI+PROG (784±32).

DISCUSSION

The results of the present study indicated that PROG up-regulates the mRNA and protein expression of neuronal BDNF in the injured spinal cord and also BDNF protein in the normal tissue. Concomitantly, steroid treatment also prevented the lesion-induced chromatolysis, supporting at

the molecular and morphological levels the neuroprotective actions of PROG.

Detection of BDNF mRNA and protein in motoneurons is in agreement with previous suggestions that motoneurons themselves are a source of neurotrophins (Dreyfus et al., 1999; Buck et al., 2000) and that locally synthesized BDNF may be an autocrine/paracrine regulator of neuronal functions (Miranda et al., 1993; Acheson and Linsday, 1996; Davies, 1996). Levels of BDNF mRNA and protein were further enhanced in rats with SCI receiving PROG, pointing out that local synthesis of BDNF is under hormonal regulatory control. In the normal spinal cord, PROG increased BDNF immunolabeling, without changes of the mRNA, suggesting that part of neuronal BDNF may originate outside the neuron. In both the normal and denervated muscle, BDNF expression is maintained throughout adult life (Clatterbuck et al., 1994; Zhang et al., 2000), representing a target-derived neurotrophic factor for adult motoneurons. Besides muscle, Schwann cells (Zhang et al., 2000), CNS glial cells (Dougherty et al., 2000) and neurons in dorsal root ganglion (Yan et al., 1994) may be potential neurotrophin donors for the normal and injured spinal cord (Dougherty et al., 2000; Zhang et al., 2000). Thus, our results raise the possibility that hormonal treatment might be increasing BDNF transport from external sources, besides increasing its synthesis in motoneurons.

A second observation was the marked reduction of both BDNF mRNA and protein expression by 3 days following SCI. Ikeda et al. (2002) and Kobayashi et al. (1997) described that although BDNF mRNA increases in neurons during an early phase of spinal cord compression injury, or in axotomized facial motoneurons, it returned to normal within 3–4 days. Here, we found that both BDNF mRNA and protein expression were down-regulated by 75 h after SCI as compared with CTL animals, a period coincident with intense chromatolytic changes and, as shown before, with depletion of choline acetyl transferase and mRNA for the Na,K-ATPase (Labombarda et al., 2002). Thus, failure to sustain the expression of BDNF may cause impairment of cell function, induce neuronal degeneration and inhibit axonal regeneration (Nakamura and Bregman, 2001).

Although survival of mature motoneurons may depend on a combination of growth factors (Oorschot and McLennan, 1998), several evidences demonstrate the central role of endogenous BDNF in providing trophic support to CNS neurons following axotomy (Giehl et al., 1998; Zhang et al., 2000), degenerative diseases (Chun et al., 2000) and ischemia (Beck et al., 1994). Recent studies found that up-regulation of BDNF and TrkB genes in motoneurons correlates with improved axonal regeneration (Al-Majed et al., 2000) and mediates neuroplasticity (Gomez Pinilla et al., 2002; Skup et al., 2002). Furthermore, studies in the BDNF knockout mice show that this neurotrophin is required for the full induction of reflex plasticity, coordination and balance (Ernfors et al., 1995), events coordinated at the spinal cord level.

A third important finding was that PROG administration to rats with SCI enhanced 200% mRNA BDNF and substantially increased neuronal BDNF protein expression and immunopositive fiber density compared with untreated animals. Again, this time period of PROG effects was coincident with repletion of choline acetyl transferase, increased levels of mRNA for the Na,K-ATPase and GAP-43 (Labombarda et al., 2002) and preservation of Nissl bodies. The finding that PROG enhanced the BDNF-immunopositive fiber network raised the possibility that the steroid may be also modulating BDNF availability to the injured spinal cord, in addition to the enhancement of BDNF mRNA and protein expression in motoneurons. It is also known that several steroids modulate the expression of neurotrophins and/or their receptors in the CNS, as shown for estrogen, PROG (Sohrabji et al., 1995; Gibbs, 1999), glucocorticoid and mineralocorticoids (Chao and McEwen, 1994; Hansson et al., 2000; Rage et al., 2002). In addition, several reports point to neurotrophic factors as possible mediators of steroid hormone action (Forger et al., 1998; Ianova et al., 2001; Solum and Handa, 2002). As previously reported, PROG could also recover the lesion-induced decrease in neuronal choline acetyltransferase indirectly by convergence with growth factors, as demonstrated in reproductive tissues (Lange et al., 1998; Peluso and Pappalardo, 1999).

Regarding the mechanisms by which PROG stimulated BDNF mRNA and protein expression, detection of intracellular PR in the spinal cord (Monks et al., 2001; Labombarda et al., 2000b) suggests a role of the classical PR in the stimulation of BDNF expression. In the case of estrogens, a putative estrogen response element in the BDNF promoter (Sohrabji et al., 1995) probably drives BDNF transcription under the control of the estrogen receptor. In contrast, glucocorticoid receptors interact with proteins of the AP-1 complex to regulate BDNF gene transcription (Hansson et al., 2000). Whether PROG effects are due to PR binding to hormone-response element or to interactions with proteins of the AP-1 complex or other transcriptional factors is presently unknown.

In the spinal cord (and other tissues as well) a putative PROG membrane binding protein called 25 Dx of still poorly defined function has been described (Labombarda et al., 2003). Since levels of 25 Dx mRNA and protein are increased after PROG administration to rats with SCI, 25 Dx may become important for PROG effects under pathological conditions. Also, the conversion of PROG to its metabolites dihydroprogesterone and tetrahydroprogesterone (Guennoun et al., 2001) may be affecting BDNF expression. These reduced derivatives modulate inhibitory and excitatory neurotransmission at the cell membrane (Majewska et al., 1986; Rupprecht et al., 1996). Since interplay between neurotransmitter receptor systems can regulate BDNF expression (Zafra et al., 1991), PROG effects may be driven through these intermediates. Finally, increased stability of BDNF protein and mRNA will result from the inhibition of oxidants and free radicals arising after SCI, since PROG prevents injury-induced lipid peroxidation (Roof et al., 1997) and exerts antioxidants effects in a murine model of spinal cord neurodegeneration (González Deniselle et al., 2003).

SCI was accompanied by typical signs of chromatolysis (Young, 1966; Tanridag et al., 1999; Nacimiento et al., 1995). Chromatolysis does not always represent a unified process but may also underlie disruptive, regulative or restorative events (Guntinas-Lichius et al., 1996). However, as a typical feature of motoneuron degeneration, chromatolysis culminates in cell dysfunction and death (Wakayama, 1992; Grossman et al., 2001; Eidelberg et al., 1989). Although the effects of PROG on cell loss following SCI were not presently investigated, the fact that in PROG-treated injured animals cells with the severe type of chromatolysis were uncommon, makes this an attractive possibility. Prospective studies will include a time course analysis of the effect of PROG on neuronal loss employing accepted markers of cell death. In the long run, enhancement of neuronal BDNF expression, reversion of severe chromatolysis and stimulation of key features of neuronal and glial cell function (Labombarda et al., 2002; De Nicola et al., 2003), suggest that PROG provides a local trophic support and represents a new approach to prevent neuronal death after injury.

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