ORIGINAL ARTICLE

Progesterone Treatment of Spinal Cord Injury

Effects on Receptors, Neurotrophins, and Myelination

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Abstract

In addition to its traditional role in reproduction, progesterone (PROG) has demonstrated neuroprotective and promyelinating effects in lesions of the peripheral and central nervous systems, including the spinal cord. The latter is a target of PROG, as nuclear receptors, as well as membrane receptors, are expressed by neurons and/or glial cells. When spinal cord injury (SCI) is produced at the thoracic level, several genes become sensitive to PROG in the region caudal to the lesion site. Although the cellular machinery implicated in PROG neuroprotection is only emerging, neurotrophins, their receptors, and signaling cascades might be part of the molecules involved in this process. In rats with SCI, a 3-d course of PROG treatment increased the mRNA of brain-derived neurotrophic factor (BDNF) and BDNF immunoreactivity in perikaryon and processes of motoneurons, whereas chromatolysis was strongly prevented. The increased expression of BDNF correlated with increased immunoreactivity for the BDNF receptor TrkB and for phosphorylated cAMP-responsive element binding in motoneurons. In the same SCI model, PROG restored myelination, according to measurements of myelin basic protein (MBP) and mRNA levels, and further increased the density of NG_2^+-positive oligodendrocyte progenitors. These cells might be involved in remyelination of the lesioned spinal cord. Interestingly, similarities in the regulation of molecular parameters and some cellular events attributed to PROG and BDNF (i.e., choline acetyltransferase, Na,K-ATPase, MBP, chromatolysis) suggest that BDNF and PROG might share intracellular pathways. Furthermore, PROG-induced BDNF might regulate, in a paracrine or autocrine fashion, the function of neurons and glial cells and prevent the generation of damage.

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Introduction

Presently, a growing list of publications gives evidence for the neuroprotective and promyelinating effects of progesterone (PROG) in the peripheral and central nervous systems (PNS and CNS, respectively). In peripheral nerves, PROG promotes remyelination attributable to injury or to the effects of old age (Koenig et al., 1995; Desarnaud et al., 1998;

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Magnaghi et al., 2001; Azcoitia et al., 2003). In the CNS, PROG stimulates myelination in organotypic slice cultures of 7-d-old rat and mouse cerebellum (Ghoumari et al., 2003), and partially reverses toxin-induced demyelination in old male rats (Ibanez et al., 2004). PROG also facilitates cognitive recovery and prevents neurodegeneration after cortical contusion (Roof et al., 1994, 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). In the spinal cord, PROG increases motoneuronal survival following axotomy (Yu et al., 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 spinal cord injury (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 neuronal Na,K-ATPase and further increases *GAP-43* mRNA levels (Labombarda et al., 2002). There is also evidence that PROG promotes remyelination and increases the number of NG_2^+ oligodendrocyte progenitors (De Nicola et al., 2003).

It is likely that multiple mechanisms operate after PROG treatment is given to animals with diverse types of CNS injury. Involvement of the classical nuclear PROG receptor (PR) in neuroprotection is suggested by the identification of both estrogen-inducible (Monks et al., 2001) and estrogen-insensitive PRs in neurons and glial cells of the rat spinal cord (Labombarda et al., 2000b). However, the presence of a membrane receptor for PROG in the spinal cord, called 25-Dx (Labombarda et al., 2003), and PROG metabolism to reduced derivatives (Guennoun et al., 2001), which modulate the activity of neurotransmitter receptors (Majewska et al., 1986; Rupprecht et al., 1996), further supports the assumption that PROG action involves pleiotropic mechanisms.

It seems important as well to elucidate the possible intermediates of PROG action. Interestingly, brain-derived neurotrophic factor (BDNF), a member of the nerve growth factor family of trophic factors, mimics some of the PROG effects in the spinal cord. For example, application of BDNF prevents the axotomy-induced decrease of choline acetyltransferase 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 (MBP) 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 that they might play an important role in neuronal survival (Thoenen et al., 1995) and axonal regeneration (Thoenen, 1995; Sayer et al., 2002). Recent data indicate 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 PRs (Labombarda et al., 2000b) and, as already stated, neurotrophins and their cognate receptors (Schober et al., 1999). Although colocalization studies of these molecules are still needed, this cellular distribution suggests that PROG modulation of motoneuron parameters might involve modulation of endogenous trophic factors.

The effects of BDNF are mediated by its cognate tyrosine-type receptor TrkB. In turn, TrkB activation activates phosphorylation cascades, the best characterized systems being the mitogen-activated protein kinase (MAPK) cascade and the PI-3K/Akt pathway (Mattson et al., 2004). In addition, it has been demonstrated that neuromodulators and trophic factors converge on cAMP-responsive element binding (CREB) protein phosphorylation (pCREB), which is a transcription factor regulating neuronal plasticity (Walton and Dragunow, 2000). It also stimulates MBP expression and myelin formation by oligodendrocytes (Afshari et al., 2001). Besides stimulating myelin formation, BDNF and pCREB inhibit apoptosis and increase the anti-apoptotic gene *bcl2*. Under pathological conditions such as SCI, stimulation of pCREB by PROG-derived BDNF would allow neuronal survival and promote remyelination following injury-induced demyelination.

PROG Effects on BDNF and pCREB in Normal and Lesioned Spinal Cord

To clarify whether endogenous BDNF expression was regulated by PROG in neurons of normal and injured spinal cord, we used *in situ* hybridization (ISH), as well as immunocytochemistry, to analyze
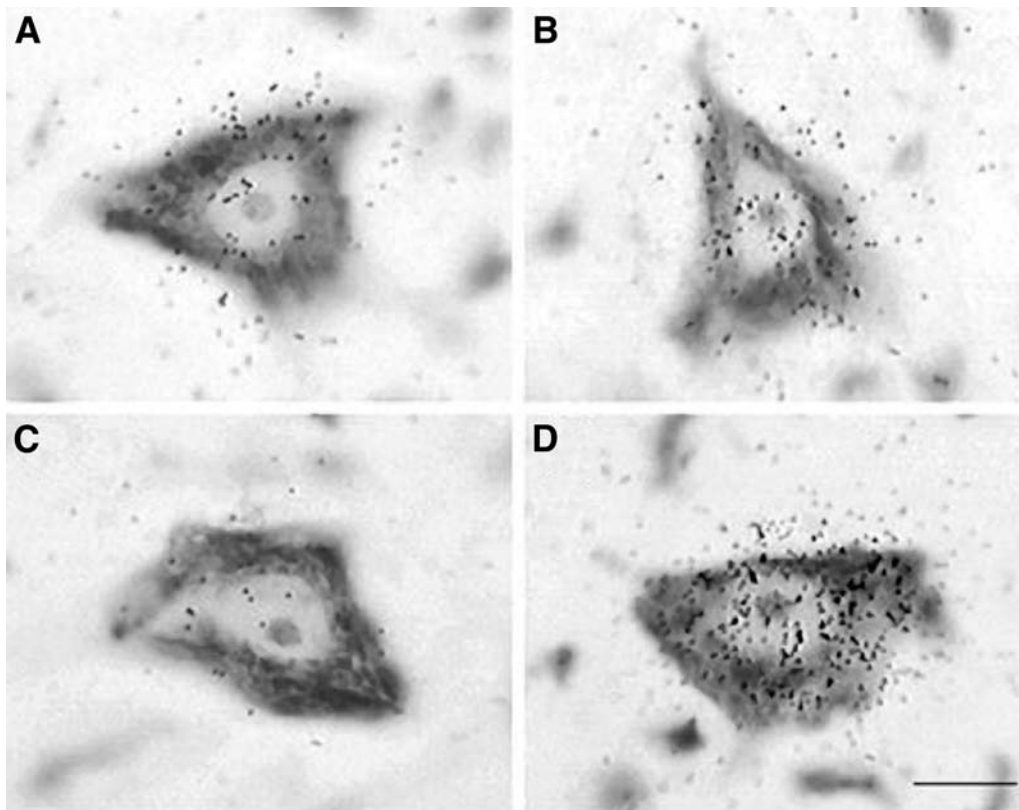
Fig. 1. Representative bright-field photomicrographs showing ISH for BDNF mRNA expression in ventral horn moto-neurons from a control rat **(A)**, a control rat receiving PROG **(B)**, a rat with SCI **(C)**, and a rat with SCI receiving PROG treatment **(D)**. The number of grains was decreased after SCI **(C)**. In turn, PROG administration to the lesioned group significantly enhanced grain density **(D)**. This plate was generated, without alterations, from digital images. Scale bar in **D** = 15 μm (also applies to **A–C**). (Reprinted, with permission, from Gonzalez et al., 2004.)

changes in BDNF mRNA and protein, respectively. In this and later studies, male Sprague-Dawley rats were sham-operated or underwent complete SCI at T10 level. PROG treatment was given as follows: rats received oil vehicle or four injections of PROG (4 mg/kg [b.w.]) at 1 h and again at 24, 48, and 72 h (sc) postlesion. 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, 2000b, 2002), and improved clinical and histological outcome after spinal cord contusion (Thomas et al., 1999). For ISH, we used a 48-mer oligonucleotide probe containing the complementary sequence to 562–609 bp of rat BDNF (Maison-pierre et al., 1991). BDNF mRNA expression was quantitated by computerized image analysis in large ventral horn neurons (>500 μm^2) of spinal cord lamina IX, considered α-motoneurons, based on size and anatomical localization. The photomicrograph of Fig. 1 summarizes the outcome of this experiment.

Quantitative analysis showed that the number of silver grains/mm^2 clustered over the neurons was similar in the control and control + PROG groups, whereas SCI reduced BDNF mRNA levels by 50% compared with control values (control, 53.5 ± 7.5 grains/mm^2, vs SCI, 27.5 ± 1.2, $p < 0.05$). However, a 3-d course of PROG treatment of the injured animals elicited a threefold 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 ($p < 0.001$).

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. For this purpose we used a commercial antibody raised against purified BDNF (N-20, sc: 546, polyclonal rabbit antiserum, Santa Cruz Biotechnology, Santa Cruz, CA). BDNF-immunopositive cells were classified, according to optical density staining intensity, into light, medium, dark, or very
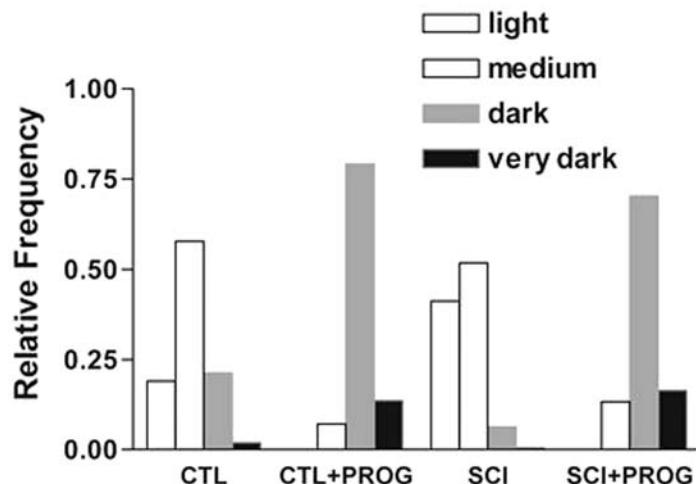
Fig. 2. Quartile distribution of BDNF immunoreactive cells in the spinal cord. Optical density scores were used to classify labeled neurons on a 4-point scale (light, medium, dark, and very dark). The relative frequency distributions of BDNF immunoreactive densities were analyzed by χ^2 test for independence, followed by partitioning analysis of contingency tables ($\chi^2 = 812.73$, $p < 0.0001$). Group labeling: CTL, control sham-operated rats; CTL + PROG, controls receiving PROG; SCI, animals with spinal cord injury; SCI + PROG, SCI group receiving PROG treatment. The distribution of label density (inverse log of grain intensity per area [ILIG/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. (Reprinted, with permission, from Gonzalez et al., 2004.)

dark. Figure 2 shows that a significant difference existed across treatment groups in the frequency distribution of BDNF-immunoreactive density of motoneurons ($\chi^2 = 812.73$, $p < 0.00001$). PROG administration to control rats shifted the density distribution to higher values (i.e., dark- and very dark-stained cells) than those observed in the control group ($p < 0.05$). In this case, 80% of neurons were classified as dark in the control + PROG group, whereas only 21% scored in this category in control rats. After SCI, density scores were shifted to lower values (i.e., light and medium staining) with respect to CTL, CTL+PROG, and SCI+PROG 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 control animals. In contrast, the SCI + PROG group showed a significant shift to higher density values owing 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. BDNF immunoreaction density of cell processes also presented group differences. After SCI, there was a dramatic loss of BDNF-positive fibers, whereas in SCI + PROG animals a plexus of heavily labeled neurites appeared. In addition,

numerous granular and intense BDNF-immunopositive deposits resembling terminal swellings (Skup et al., 2002) were detected in apposition to neuronal perikarya.

To investigate if PROG effects on BDNF also involve pCREB, neuronal expression of this transcription factor was investigated by immunocytochemistry. In animals with SCI, nuclear immunolabeling for pCREB was reduced as compared with control motoneurons (33% reduction, $p < 0.01$). Application of our standard PROG protocol treatment to SCI rats produced a threefold increment in nuclear pCREB ($p < 0.001$ vs untreated SCI). These findings suggest that PROG stimulation of BDNF gene transcription might lead to accumulation of pCREB in neuronal nuclei, although the pathways and kinases involved in this effect have not yet been elucidated. Nevertheless, it shows that pCREB is probably involved in the neuroprotective effects of PROG.

PROG Regulation of Chromatolysis

Chromatolysis is a typical incident of injured neurons and represents the loss of cytoplasmic ribonucleoproteins. We studied whether PROG neuroprotection after SCI inhibited this process and preserved Nissl staining. First, we observed that motoneurons from control and control + PROG
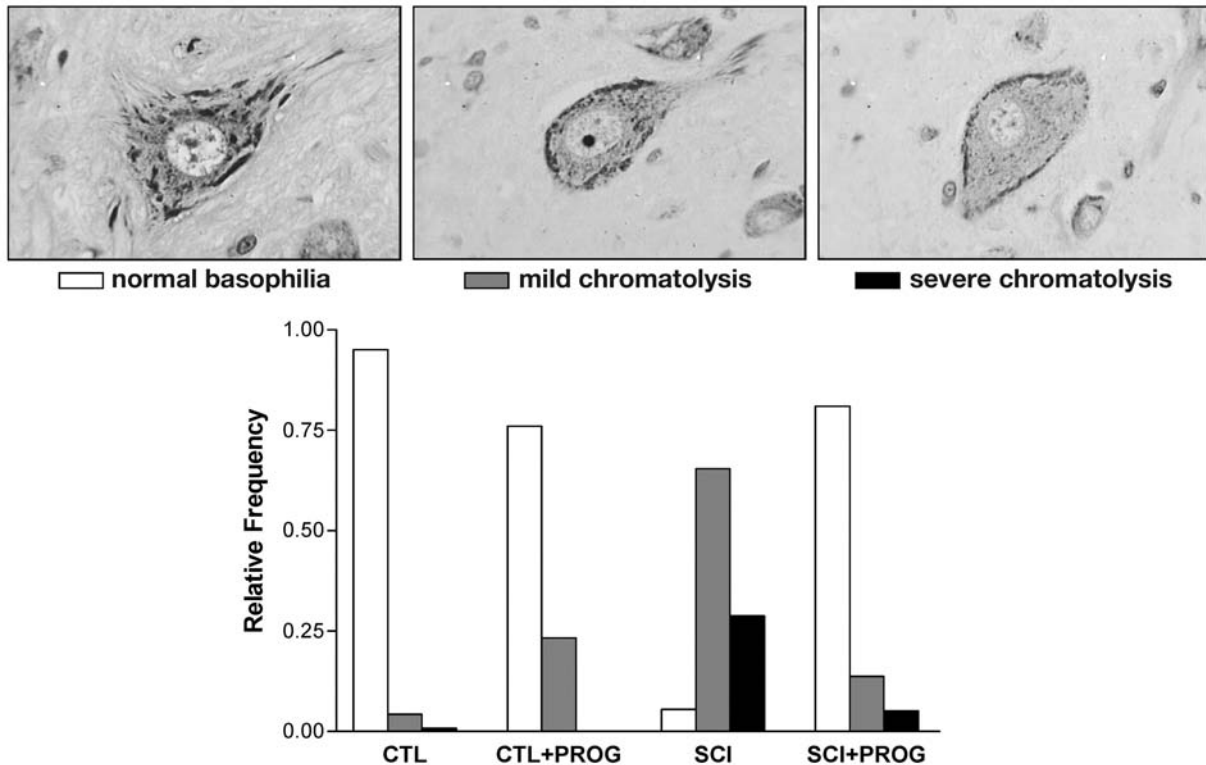
Fig. 3. (Bottom) Frequency histograms showing different motoneuron phenotypes in CTL, CTL + PROG, SCI, and SCI + PROG groups (group labeling as in the legend to Fig. 1). (Top) Representative cresyl violet–stained motoneurons were selected to show the 3-point scale classification: normal basophilia (left), mild chromatolysis (center), or severe chromatolysis (right). After SCI, only 5% of motoneurons appeared normal and 30% of motoneurons corresponded to the severe phenotype, showing eccentric nucleus, dissapearance 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 a few cells showed mild chromatolytic changes (Reprinted, with permission, from Gonzalez et al., 2004.)

animals showed a normal basophilia and were characterized by clusters of Nissl bodies in multiple locations throughout the cytoplasm (Fig. 3, left-hand photograph). Following SCI, most motoneurons were mildly chromatolytic (Fig. 3, middle photograph) or presented the severe type (Fig. 3, 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 frequency histograms (Fig. 3) demonstrated that significant differences existed among the experimental groups (χ^2 = 210.53, $p <$ 0.0001). After SCI, most motoneurons scored as mild (65%) or severe (30%) chromatolytic degeneration ($p < 0.001$ vs CTL). In the SCI + PROG animals, Nissl staining appeared normal in 81% ventral horn neurons, whereas just a minority showed the mild (14%) or severe type (5%) of chromatolysis ($p < 0.001$ vs

SCI). Thus, chromatolytic degeneration was prevented by PROG treatment of rats with SCI.

PROG Effects on Expression of MBP and Density of Oligodendrocyte Precursor Cells in Normal and Injured Spinal Cord

MBP is a component of central myelin, which provides a reliable method for assessing the process of myelination in the brain (Hamano et al., 1996; Muse et al., 2001). Considering that MBP expression responded to PROG treatment in several experimental models (Jung-Testas et al., 1996; Ghoumari et al., 2003; Ibanez et al., 2004), we studied if PROG prevented SCI-induced demyelination. To this end, we employed a monoclonal MBP antibody (Boehringer, Mannheim, Germany) and immunocytochemical techniques to determine the immunoreaction staining intensity of the corticospinal tract (CST), the dorsal ascending tract (DAT),

and the ventral funiculus (VF) in controls rats with and without PROG treatment and in similarly treated rats with SCI. The areas of white matter were selected considering that after SCI, CST fibers in the lumbar region below the lesion site represented axons undergoing axonal degeneration; those in DAT, the reaction of proximal axons; and VF staining was the response of ventral roots originating in motoneurons. In all cases, MBP staining was diffuse, without labeling of individual cells. SCI led to a pronounced depletion of MBP immunostaining in CST and DAT. When given to rats with SCI, PROG maintained MBP staining near control levels in both CST and DAT. In contrast to findings in dorsal white matter, staining intensity in VF remained unchanged after SCI or PROG treatment, indicating that the steroid response was region specific. To investigate if changes in MBP immunostaining were also reflected at the mRNA level, ISH was carried out using a probe specific for MBP exon1 mRNA (Sim et al., 2000). Films of auto radiograms of the SCI + PROG group demonstrated a stronger hybridization signal in areas of the dorsal funiculus (CST + DAT) compared with the other groups, confirming that PROG stimulation of MBP mRNA and protein required a tissue sensitized previously by SCI, as the hormone was without effect in control, sham-operated rats.

To explain the enhanced MBP immunostaining of rats with SCI receiving PROG, we hypothesized that PROG might stimulate oligodendrocyte development and differentiation, as oligodendrocyte precursor cells (OPCs) are able to produce the myelin proteins MBP, myelin-oligodendrocyte protein, cyclic nucleotide phosphodiesterase (CNPase), and proteolipid protein (Ye et al., 2003; Li and Blakemore, 2004). Oligodendrocyte precursor cells (OPCs) were labeled with an antibody recognizing the NG_2 proteoglycan (gift of W. B. Stallcup, The Burnham Institute, La Jolla, California), and the number of NG_2^+ cells per 200 mm^2 was determined in longitudinal sections of the gray and white matter below the lesion site. These cells were practically absent in control rats receiving vehicle or PROG treatment but in agreement with others (Nishiyama et al., 1999; Levine et al., 2001; Hubbard, 2003); SCI alone stimulated NG_2^+ cell number over controls in white matter and gray matter. PROG treatment of rats with SCI dramatically increased NG_2^+ cell density in both gray and white matter, as compared with CTL and SCI groups receiving vehicle, suggesting that the steroid effects on remyelination could be partly explained by an action on OPCs.

PROG Receptor(s) in Control and Injured Spinal Cord

As pointed out in the Introduction, an estrogen-insensitive PR recently has been 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 were found to be PR positive, using an antibody recognizing the B-form of PR. Whereas in cells of the pituitary gland and uterus the PR is exclusively nuclear, in spinal cord neurons and glial cells PR staining is also present in the cytoplasm and cell processes (Labombarda et al., 2000b). We have investigated the presence and regulation of PR expression using semiquantitative reverse transcription polymerase chain reaction (RT-PCR) and immunocytochemistry (Labombarda et al., 2003). The aim was to describe the response of PR to injury and hormone treatment. For RT-PCR, forward and reverse primers derived from nucleotides 1565–1586 and 1928–1907 of the rat PR cDNA sequence (Labombarda et al., 2003) were used for amplification of a 380-bp fragment. The temporal profile of PR mRNA expression after SCI showed that PR expression declined rapidly to 34% at 6 h after SCI when compared with control animals and remained below control levels at 24 h (66%) and 72 h (55%). Thereafter, rats treated with PROG or naïve animals were killed 72 h after SCI. The reduction attributable to SCI was not modified by PROG treatment (Fig. 4). Thus, PROG was without effect on the reduced levels of PR mRNA caused by spinal cord trauma. The regulation pattern of PR gene expression at the protein level paralleled that observed for mRNA levels. SCI decreased PR immunostaining intensity of both motoneurons and glial cells, whereas PROG treatment did not restore PR protein expression.

In subsequent experiments we studied the response to SCI and PROG treatment of the membrane PROG-binding site called 25-Dx. Site 25-Dx is similar to a putative membrane receptor for PROG cloned from porcine vascular smooth muscle cells (Falkenstein et al., 1996, 1999) and humans (Gerdes et al., 1998). The rat homolog of this molecule, encoding a 223-amino-acid peptide (25-Dx) (Selmin et al., 1996) is highly expressed in hypothalamus (Krebs et al., 2000). 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 cloned from the rat CNS (Runko et al., 1999) of hitherto unrelated functions.
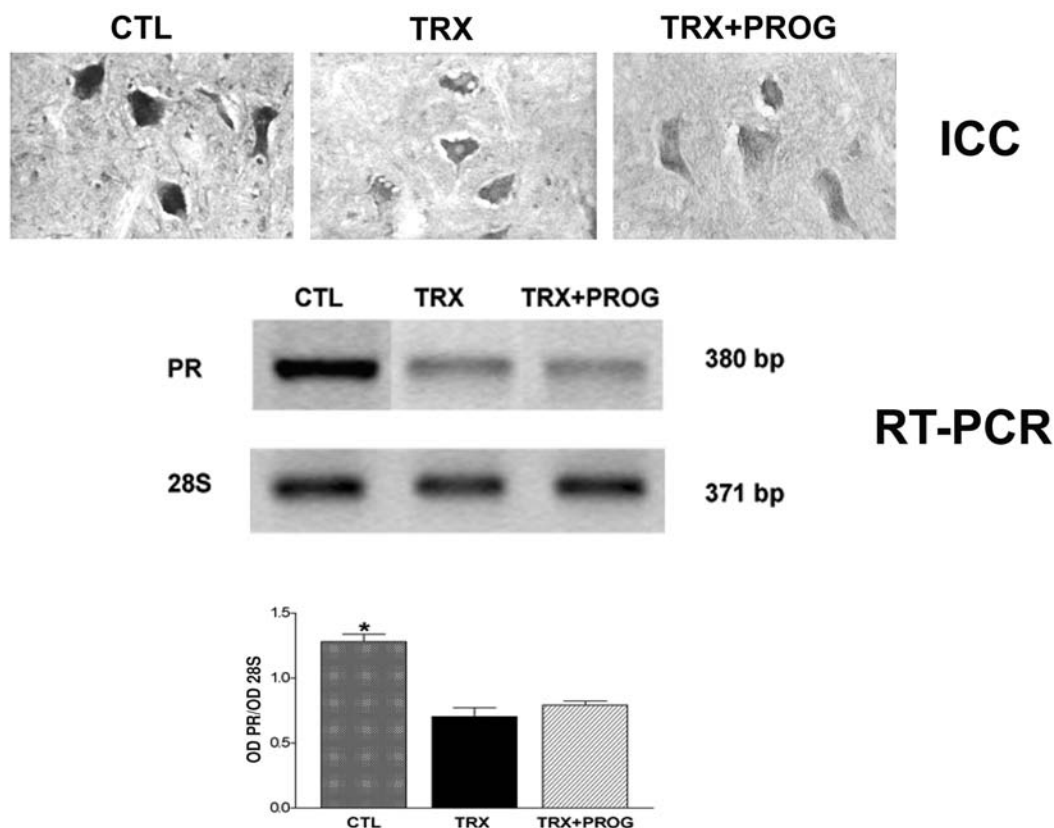
Fig. 4. Effects of transection (TRX) and PROG treatment on PR expression in the spinal cord. The upper three photomicrographs represent PR-immunoreactive motoneurons in a control (CTL), a transected rat (TRX), and a transected rat given progesterone (TRX + PROG). The middle bands represent PR mRNA in three groups; semiquantitative data for RT-PCR are presented in the lower graph. It is shown clearly that TRX injury (SCI) decreases both PR mRNA and protein, whereas PROG does not regulate this receptor in injured animals (Reprinted, with permission, from Labombarda et al., 2003.)

For RT-PCR of 25-Dx, forward and reverse primers corresponded to nucleotides 370–389 and 711–692 of rat 25-Dx (accession no. U63315) and PROG receptor membrane component 1 (accession no. NM_021766) published sequences (Selmin et al., 1996); they were expected to amplify a cDNA fragment of 341 bp. In the spinal cord of control animals, abundance of 25-Dx mRNA represented 26% of levels present in the hypothalamus. In disparity with the profile expression of PR mRNA, no significant changes were observed for 25-Dx mRNA levels at 72 h after injury. However, and in contrast to data for PR mRNA, PROG upregulated 25-Dx expression in the injured spinal cord. Thus, in the SCI group receiving PROG, 25-Dx mRNA was 86% higher than in controls and 57% higher than in the SCI-only group.

Unlike the cytoplasmic and nuclear localization of PR immunostaining of neurons and glial cells, staining with the IZAg antibody (recognizing 25-Dx protein) was exclusively neuronal, showed preferential staining of the dorsal horn and central canal neurons, and was prominent in plasma membranes. Glial cells of the white or gray matter were negative for IZAg immunostaining. Therefore, cellular as well as subcellular distribution of PR and 25-Dx were quite different. Site 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. Furthermore, after SCI, the number of IZAg-immunolabeled cells decreased by 28.5%, but treatment with PROG increased the number of positive cells. The opposite effects of PROG treatment on PR and 25-Dx, including expression levels of their respective mRNAs and proteins, suggest a dissimilar role played by these molecules after injury and steroid treatment.

Conclusions

Data summarized in this review indicate that PROG upregulated 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 increased pCREB immunoreactivity in motoneuron cell nuclei and prevented lesion-induced chromatolysis, supporting the neuroprotective actions of PROG at the molecular and morphological levels. Detection of BDNF mRNA and protein in motoneurons (Dreyfus et al., 1999; Buck et al., 2000), coupled with the beneficial effects of this neurotrophin on damaged motoneurons, indicates that locally synthesized BDNF might 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 might originate outside the neuron. Our results raise the possibility that hormonal treatment might be increasing BDNF transport from external sources, besides increasing its local synthesis in motoneurons.

A second observation was that both BDNF mRNA and protein expression were downregulated by 75 h after SCI as compared with control animals, a period coincident with intense chromatolytic changes and, as shown previously, with depletion of choline acetyltransferase and mRNA for Na,K-ATPase (Labombarda et al., 2002). Thus, failure to sustain the expression of BDNF might cause impairment of cell function, induce neuronal degeneration, and inhibit axonal regeneration (Nakamura and Bregman, 2001). Evidence demonstrates the central role of endogenous BDNF in providing trophic support to CNS neurons. Rescuing axotomized corticospinal neurons by glial-derived neurotrophic factor requires the presence of endogenous cortical BDNF (Giehl et al., 1998), and deprivation of BDNF impairs myelination of regenerating axons (Zhang et al., 2000). Recent studies found that upregulation 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 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.

It is also important to point out 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 acetyltransferase, increased levels of mRNA for 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 also might be modulating BDNF availability to the injured spinal cord, in addition to the enhancement of BDNF mRNA and protein expression in motoneurons. Whether PROG effects on BDNF are direct or indirect remains a mystery. 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 attributable to PR binding to hormone-response elements on the BDNF gene or to interactions with proteins of the AP-1 complex or other transcriptional factors is presently unknown.

SCI was accompanied by typical signs of chromatolysis (Nacimiento et al., 1995; Young, 1966; Tanridag et al., 1999), a feature of incipient motoneuron degeneration that culminates in cell dysfunction and death (Eidelberg et al., 1989; Wakayama, 1992; Grossman et al., 2001). PROG significantly prevented the lesion-induced chromatolysis of spinal neurons, as a significant number of neurons from the SCI + PROG group presented normal Nissl staining. 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 local trophic support and represents a new approach to prevent neuronal death after injury.

Another objective of our investigation was to elucidate whether PROG neuroprotection after spinal cord transection injury involved, in addition to motoneurons, the regulation of myelin proteins, myelin precursors, and myelin-producing cells. We were able to show that spinal cord lesion reduced MBP mRNA, and protein expression in axons damaged retrogradely (DAT) and anterogradely (CTS). Others have observed that spinal cord lesion produces loss of myelin proteins in white matter tracts (Bresnahan, 1978; Bunge et al., 1993; McTigue et al.,

1998). In contrast, MBP showed no changes in the VF, which comprised axons of the ventral roots not deafferented by the lesion. In DAT and CST, PROG successfully maintained the levels of expression of MBP. An additional important finding regarding remyelination was that PROG effects on MBP were accompanied by changes in OPCs, determined by the NG_2 antibody. In agreement with previous reports, NG_2^+ cells were very scarce in the control spinal cord but highly populated in the lesioned tissue (Nishiyama et al., 1999; Levine et al., 2001; Hubbard, 2003). However, the low expression of MBP in rats with SCI suggests that the newly generated NG_2^+ cells did not provide enough oligodendrocytes to compensate for the failure to maintain myelination. Perhaps, the PROG stimulus was necessary to overload the damaged tissue with precursors. However, in addition to stimulating NG_2^+ division and reactivity, PROG might increase precursor differentiation and/or myelin synthesis by NG_2^+ cells; however, further experiments are needed to ascertain this issue.

NG_2^+ and OPC cells labeled with other markers are able to migrate, proliferate, and differentiate into mature oligodendrocytes and, as already pointed out, can express several myelin constituents (Nishiyama et al., 1999; Ishii et al., 2001; McTigue et al., 1998; Ye et al., 2003; Li and Blakemore, 2004). Therefore, PROG action on NG_2^+ cell density might account for the hormone's properties in maintenance of myelin proteins in the damaged spinal cord. After demyelination, mature oligodendrocytes play a minor role in myelin repair and remyelination derive in large proportion from endogenous OPCs (Keirstead and Blackemore, 1997; Carrol et al., 1998; Levine et al., 2001; Ibanez et al., 2004). Thus, it is possible that PROG increases the survival of progenitors by preventing their apoptosis, a possibility that needs further experimentation.

The effect of PROG on BDNF is tightly connected to remyelination. BDNF is a key regulator of myelin proteins in the PNS and the CNS (Chan et al., 2001; Du et al., 2003; Tolwani et al., 2004), and this property also applies to the contused spinal cord, in which MBP expression is highly enhanced by BDNF treatment (McTigue et al., 1998; Ikeda et al., 2002). The similarities in the regulation of myelin proteins attributed to BDNF and those shown here for PROG suggest that BDNF and PROG actions share common intracellular pathways regulating myelin-producing cells. The latter are fundamental players in this scenario, as they are sensitive to PROG and also take up and produce BDNF (Jung-Testas et al., 1999;

Dougherty et al., 2000; McTigue et al., 1998; Schumacher et al., 2000). In this view PROG-induced BDNF might act in a paracrine or autocrine fashion to positively regulate the function of neurons and other cell types such as oligodendrocytes. The possibility for a PROG effect on oligodendroglia receives support from recent findings in the cerebellum, in which PROG stimulates the proliferation and maturation of OPCs (Ghoumari et al., 2005).

PROG treatment of animals with SCI also increased pCREB immunoreactivity in the nuclei of motoneurons. Phosphorylation of CREB has been shown to be a necessary condition for neuronal survival and synaptic plasticity (Finkbeiner et al., 1997; Walton and Dragunow, 2000). According to Afshari et al. (2001), CREB also might be a mediator of growth factor signals that play functions in the maturation of oligodendrocytes and on the expression of MBP isoforms. Thus, a dual role for pCREB can be envisaged during PROG effects in the injured spinal cord: First, as a stimulator of neuronal survival and inductor of downstream survival genes (Walton and Dragunow, 2000), pCREB might prevent the chromatolytic degeneration of deafferented neurons. Our observations of increased pCREB immunoreactivity in motoneurons from rats with SCI receiving PROG treatment might be related to this effect. Second, it might also be involved in the stimulation of MBP and/or in the increased density of NG_2^+ cells in PROG-treated injured rats. Although evidence on whether PROG regulates BDNF and pCREB in oligodendroglial cells is lacking, preliminary experiments suggest that pCREB is more abundant in nuclei from glial cells of rats with SCI receiving PROG treatment.

It also seems important to consider the mechanisms by which PROG increased neuronal gene expression and positively regulated MBP expression after injury. The detection of PRs in the spinal cord (Labombarda et al., 2000, 2003) suggests a role of the classical receptor in many of the PROG effects in this tissue. In association with this possibility, a modulatory site for steroids, including PROG, was found in the 5'-untranslated region of the MBP gene (Verdi and Campagnoni, 1990). However, PROG might also bind to the membrane-binding protein 25-Dx and other recently cloned membrane receptors (Labombarda et al., 2003, Zhu et al., 2003), suggesting that alternative mechanisms might operate under normal and/or pathological conditions. In male rats with complete SCI, levels of PR mRNA were decreased significantly, whereas those of 25-Dx mRNA

remained unchanged with respect to control animals. When spinal cord-injured animals received PROG 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. Because the two binding proteins for PROG differed with respect to their response to lesion, hormonal regulation, and cellular and subcellular localizations, their functions might differ under normal and pathological conditions. These observations point to a novel and potentially important role of the PROG-binding protein 25-Dx after injury of the nervous system and suggest that the neuroprotective effects of PROG might involve classical as well as distinct membrane-binding sites. In addition, administered PROG is reduced to the derivatives 5α-dihydroprogesterone and 3α,5α-tetrahydroprogesterone, which promote myelination and neuroprotection in the PNS and CNS (Azcoitia et al., 2003; Ciriza et al., 2004). These demonstrations support that PROG effects in the spinal cord are pleiotropic and can be achieved via different mechanisms involving different receptors.

Acknowledgments

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