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Progesterone neuroprotection in spinal cord trauma involves up-regulation of brain-derived neurotrophic factor in motoneurons☆

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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. Both types of cells express classical intracellular PROG receptors (PR), while neurons additionally express the PROG membrane-binding site called 25-Dx. In motoneurons from rats with spinal cord injury (SCI), PROG restores to normal the deficient levels of choline acetyl-transferase and of α3 subunit Na,K-ATPase mRNA, while levels of the growth associated protein GAP-43 mRNA are further stimulated. Recent studies suggest that neurotrophins are possible mediators of hormone action, and in agreement with this assumption, PROG treatment of rats with SCI increases the expression of brain-derived neurotrophic factor (BDNF) at both the mRNA and protein levels in ventral horn motoneurons. In situ hybridization (ISH) has shown that SCI reduces BDNF mRNA levels by 50% in spinal motoneurons, while PROG administration to injured rats (4 mg/kg/day during 3 days, s.c.) elicits a three-fold increase in grain density. In addition to enhancement of mRNA levels, PROG increases BDNF immunoreactivity in perikaryon and cell processes of motoneurons of the lesioned spinal cord, and also prevents the lesion-induced chromatolytic degeneration of spinal cord motoneurons as determined by Nissl staining. Our findings strongly indicate that motoneurons of the spinal cord are targets of PROG, as confirmed by the expression of PR and the regulation of molecular parameters. 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. Thus, PROG treatment constitutes a new approach to sustain neuronal function after injury.

Keywords: Progesterone; Spinal cord injury; Brain-derived neurotrophic factor; Progesterone receptor; Neuroprotection

1. Introduction

Traumatic spinal cord injury (SCI) constitutes a devastating event that often results in complete loss of motor and sensory function [1]. Neurons, especially ventral horn motoneurons, show early degeneration and chromatolysis, with death occurring by necrosis or apoptosis depending on the severity of the lesion [2]. Several strategies have been developed to preserve neuronal function and repair damage, including transplant of peripheral nerves, olfactory ensheating cells, stem cells or schwann cells and enhancement of axonal growth using fibronectin conduits [3]. Pharmacological approaches have also been employed, such as delivery of neurotrophic factors, antioxidant compounds, antiglutamatergic drugs and steroids [3–5].

Steroid hormones offer promising therapeutic perspectives during the acute phase of spinal cord injury, since they show protective effects on damaged neurons [5]. Glucocorticoids are strongly effective for recovery of patients with spinal cord trauma and in contusion and transection models in

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rats [6]. However, gonadal steroids including 17β-estradiol and progesterone (PROG) also provide neuroprotection as shown earlier in lesions of the brain and stem motor nuclei and more recently in the spinal cord [7]. Thus, PROG prevents neuronal loss following contusion, ischemia and edema of the brain, and preserves neurons after section of the hypoglossal and facial motor nuclei [8–10]. In the spinal cord, treatment of rats with PROG increases motoneuron survival after axotomy or injury, protects cultured neurons against glutamate toxicity and normalizes defective functional parameters of injured neurons [11–13].

In rats with spinal cord injury due to transection (TRX), we have shown that deafferentiation reduces the levels of choline acetyl-transferase (ChAT) and α3 subunit mRNA of the Na,K-ATPase, while moderately up-regulates the mRNA of the growth-associated protein GAP-43 [13]. In vivo PROG treatment during 72 h restores levels of the sodium pump mRNA and ChAT to normal, whereas levels of GAP-43 mRNA are further enhanced. These responses are interpreted as protective and regenerative for the damaged tissue, in conjunction with neuronal effects, PROG strongly influences myelin synthesis in the peripheral and central nervous system (CNS) [14–17]. In the oligodendrocytes, the myelin-producing glia of the CNS, PROG increases myelination in culture and in cerebellum, as shown by the increased expression of the myelin basic protein (MBP) [15,17]. A similar effect may also take place in the spinal cord when rats with TRX receive systemic PROG treatment, although further studies are needed to define this point [18].

2. Expression of PROG receptors in the spinal cord

Both spinal cord motoneurons and glial cells express PROG receptors (PR). Immunocytochemistry using the KC 146 monoclonal antibody recognizing the B-form of PR, demonstrated that not only neurons from ventral horn and Lamina IX, but also glial cells in gray and white matter and ependymal cells are PR-positive [19]. Evidence for estrogen-inducibility of PR in ovariectomized rats or gender differences in neuronal PR immunostaining intensity is not obtained in the spinal cord. In contrast, the anterior pituitary and uterus from estrogenized female rats show the expected estrogen-dependency and a strict nuclear localization [19]. However, in neurons and glial cells of the spinal cord, PR are localized in cytoplasm and/or nucleus and in some cell processes, suggesting alternative mechanisms of hormone action.

We have also obtained evidences for the classical PR and a recently discovered PROG membrane-binding site called 25-Dx [20,21] using RT-PCR to determine the relative mRNA levels, and immunocytochemistry to establish the cellular localization of both molecules (Fig. 1). In male rats with spinal cord TRX, levels of PR mRNA significantly decreased, while mRNA of 25-Dx are unchanged respect of control animals. When spinal cord-injured animals receive PROG treatment during 72 h, PR mRNA levels remain similar to non-treated animals, while 25-DX mRNA levels are significantly increased. Immunostaining of PR show intracellular localization in neurons and glial cells, whereas 25-DX immunoreactivity localized to plasma membrane of dorsal horn

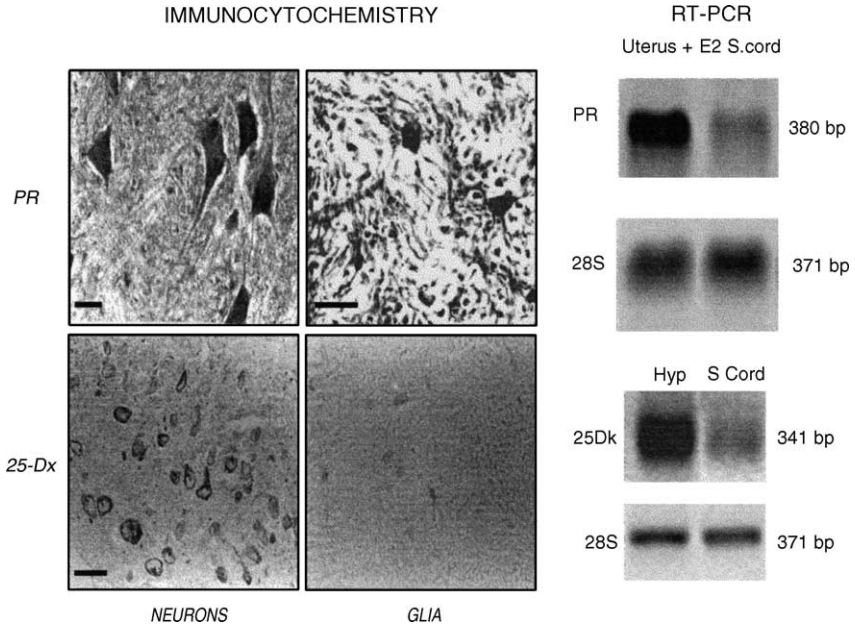

Fig. 1. Expression of progesterone receptors in the spinal cord. Left-hand panel: immunocytochemistry data show that the classical progesterone receptor (PR) is expressed in neurons and glial cells, whereas the 25-Dx progesterone binding site is found in neurons but not glial cells. Right-hand panel: RT-PCR analysis demonstrated the presence of both PR and 25-Dx mRNA in the spinal cord (S. cord), amounting to 20–30% of the maximum values found in the uterus of ovariectomized-estrogen primed rats in the case of PR (uterus + E2) and in hypothalamus (Hyp) regarding 25-Dx (modified from [20]).

and central canal neurons (Fig. 1). Since the two binding systems for PROG differ in their response to lesion, hormone treatment and regional localization, their function may also differ under normal and pathological conditions [20]. However, other mechanisms besides PR and 25-Dx may also account for PROG effects. Recently a membrane receptor for PROG (mPR) was cloned in fish and the brain of mammals [22]. Second, PROG is extensively metabolized to reduced derivatives such as 3α,5α-tetrahydroprogesterone [23] which modulate the activity of neurotransmitter receptors [24]. Evidently, multiple mechanisms can account for PROG effects in the spinal cord.

3. Neurotrophic factors, spinal cord and PROG

Brain-derived neurotrophic factor (BDNF), a member of the nerve growth factor family of trophic factors, mimics some of the PROG effects on the spinal cord. For example, application of BDNF prevents the axotomy-induced decrease of choline acetyl-transferase in motoneurons, stimulates sprouting of cholinergic fibers and hindlimb stepping and increases the expression of the regeneration-associated gene GAP-43 after spinal cord injury [25–27]. Additionally, BDNF administration decreases edema formation [28] and promotes the recovery of myelin-basic protein after compression-induced spinal cord injury [29]. Neurotrophic factors and their receptors are present not only in developing but also in adult spinal cord neurons, indicating they may play an important role for neuronal survival and axonal regeneration [30].

There are evidences supporting that steroid hormones interplay with neurotrophins in the CNS. As already shown in Fig. 1, motoneurons of the spinal cord express PR [19]. They also express neurotrophins and their cognate receptors [31]. Although colocalization studies are lacking, this cellular distribution suggests that PROG modulation of motoneuron parameters may involve the endogenous trophic factors. Indeed, we have recently shown that expression of BDNF mRNA and protein in motoneurons is under modulatory control by PROG in the injured spinal cord [32].

4. Materials and methods

In order to study PROG effects, we used Sprague–Dawley male rats (250–300 g) which underwent complete spinal cord transection at thoracic level T10 [20,32]. Sham-operated rats were not transected. For PROG treatment, four injections of 4 mg/kg PROG dissolved in vegetable oil was given at times 1 h (i.p.), and again at 24, 48, and 72 h (s.c.) post-lesion. This PROG dose prevented neuronal degeneration and loss after brain injury and was able to modulate motoneuron parameters after spinal cord injury [32]. Animals were used for the different experiments 75 h after sham surgery or TRX, and 3 h after receiving the last injection.

In situ hybridization was carried with spinal cord sections obtained from the L1 spinal level below the lesion site or a similar segment of sham-operated rats [20,32]. Sections were hybridized to a 48-mer (^{35}S)dATP-labeled synthetic oligonucleotide probe containing the complementary sequence to bp 562–609 of rat BDNF [33]. Semiquantitative analysis was performed by computer-assisted image analysis (Bioscan Optimas VI). For immunocytochemistry, sections were exposed to a primary antibody raised against purified BDNF (N-20, s.c.: 546, polyclonal rabbit antiserum, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Staining intensity and immunoreactive cell area (μm^2) were determined for each motoneuron of Lamina IX by computer-assisted image analysis [13].

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. [34]. 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. The relative frequency distribution of intensity was analysed by χ^2 test for independency. A significant difference in the overall χ^2 was followed by partitioning analysis of contingency tables. In addition, BDNF-immunopositive fiber density was quantified by image analysis (Optimas, Bioscan VI) and expressed as BDNF-immunopositive fiber density (μm tmmunopositive fiber length/30 mm^2), following the method of Skup et al. [35].

To study the effects of TRX and PROG treatment on chromatolysis, cresyl violet stained neurons were classified as normal, “mild” chromatolytic and “severe” chromatolytic, according to previously reported criteria [36, 37].

5. PROG effects on BDNF mRNA and protein expression

In control animals, BDNF mRNA and protein were expressed in large ventral horn neurons (>500 μm^2) of Rexed Lamina IX, considered α-motoneurons based on size and anatomical localization. A marked reduction of both BDNF mRNA and protein expression was found by 3 days following SCI. Ikeda et al. [29] and Kobayashi et al. [27] 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 to control animals, a period coincident with intense chromatolytic changes (see below) and, as shown before, with depletion of choline acetyltransferase and the α3 subunit mRNA of Na,K-ATPase [13]. Thus, failure to sustain the expression of BDNF may

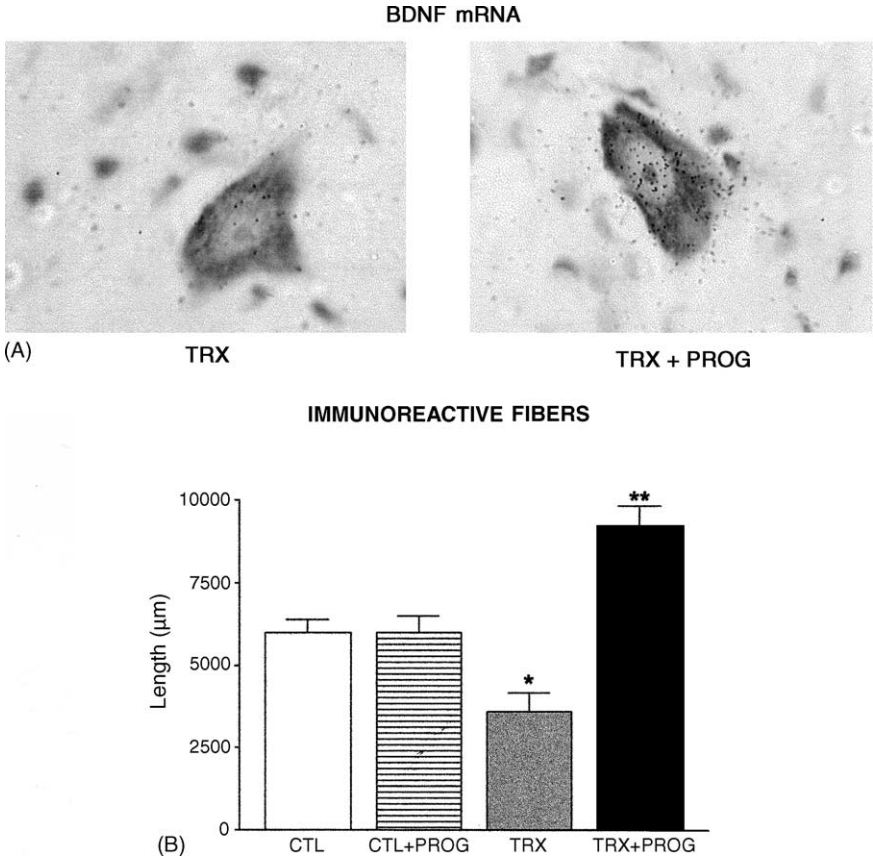

Fig. 2. (A, upper graph): in situ hybridization shows low expression of BDNF mRNA in rats with spinal cord transection (TRX), which are stimulated in rats with TRX receiving progesterone (TRX + PROG). (B, lower graph): length of BDNF-immunoreactive motoneuron fibers in control rats (CTL), controls receiving progesterone (CTL + PROG), rats with transection (TRX) and lesioned rats receiving progesterone (TRX + PROG): (*) TRX vs. CTL and CTL + PROG: $p < 0.05$; (**) TRX vs. TRX + PROG: $p < 0.001$ (ANOVA and post-hoc Bonferroni's test).

cause impairment of cell function, induce neuronal axonal regeneration, as previously, suggested by Nakamura et al. [38].

An important finding was that PROG administration to rats with spinal cord injury enhanced 200% mRNA BDNF and substantially increased neuronal BDNF protein expression and immunopositive fiber density compared to untreated animals (Fig. 2). 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 [13] and preservation of Nissl bodies (see below). 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.

6. PROG effects on chromatolysis

Spinal cord injury is followed by signs of chromatolysis, as previously reported by several groups [37,39]. As a typical feature of motoneuron degeneration, chromatolysis culminates in cell dysfunction and death [40–42]. PROG significantly prevented the lesion-induced chromatolysis of spinal neurons, since a significant number of neurons from the spinal cord injury group receiving PROG presented normal Nissl staining. Control motoneurons (with or without PROG treatment) were characterized by clusters of Nissl bodies in multiple locations throughout the cytoplasm. Following injury, most motoneurons were mild chromatolytic, or presented the severe type, consisting in 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. 3), demonstrated that significant differences existed among the experimental groups ($\chi^2 = 210.53$, $p < 0.0001$). After injury, only 5% neurons remained normal, and most motoneurons scored as mild (65%) or severe (30%) chromatolytic ($p < 0.001$ versus control). In the injury group treated with PROG, Nissl staining appeared normal in 81% ventral horn neurons, whereas just a minority showed mild (14%) or the severe type (5%) of chromatolysis ($p < 0.001$ versus injury vehicle).

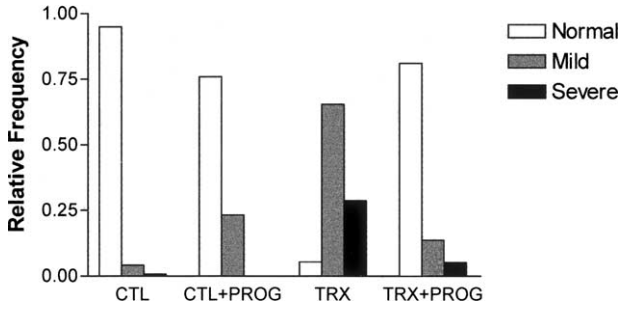

Fig. 3. Frequency histograms showing the distribution of chromatolytic phenotypes in motoneurons from controls (CTL), CTL + PROG, spinal cord transection (TRX) and TRX + PROG groups. In the histogram, empty columns denote cells with normal basophilia, gray columns those with mild chromatolysis and dark columns represent cells with severe chromatolysis. After transection, only 5% of motoneurons appeared normal and 30% of motoneurons correspond to the severe phenotype ($p < 0.001$ vs. CTL). In the TRX + PROG group, the normal pattern appeared in 81% of neurons, and only few cells showed mild chromatolytic changes ($p < 0.001$ vs. TRX).

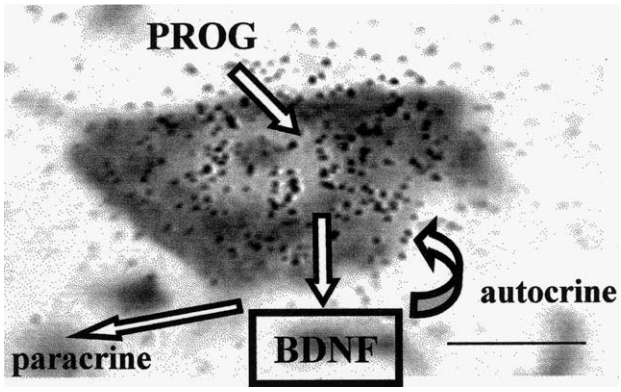

Fig. 4. Progesterone induces BDNA expression in motoneurons, which acts in an autocrine manner in the BDNF-producing cell or in a paracrine fashion upon neighboring neurons or glial cells.

7. Conclusions

Our data demonstrated that in rats with TRX, PROG induced an up-regulation of BDNF mRNA and protein. These PROG effects on motoneurons may be supportive of neuronal recuperation. As previously shown, deafferented motoneurons from animals with spinal cord lesions present several biochemical abnormalities, involving the acetylcholine-synthesizing enzyme, the sodium pump mRNA, and the GAP 43 mRNA [13]. These parameters are reverted to normal after PROG treatment is given to TRX animals, with the exception of GAP 43 mRNA which is further enhanced. As further evidence for PROG neuroprotection, the chromatolytic profile typical of degenerating neurons from rats with TRX, was considerably prevented when the animals received an intensive PROG treatment. Thus, PROG effects on biochemical markers go in parallel with morphological evidences of survival of damaged motoneurons, favoring the view that PROG supported spinal cord function by an action on neurons. Interestingly, there are similarities in the regulation of molecular parameters and some cellular events attributed to PROG and those shown for BNDF (Table 1), suggesting that BDNF and PROG actions may share common intracellular pathways in prevention of neuronal damage. Furthermore, it also suggest that BDNF may be an intermediate in PROG action. In this view, PROG-induced BDNF may act in a paracrine or autocrine fashion to positively regulate the function of neurons and perhaps other cell types, such as oligodendrocytes (Fig. 4).

It seems also important to consider the mechanisms by which PROG stimulated BDNF mRNA and protein expression. The detection of PR in the spinal cord [19,20] suggests a role of the classical PR in the stimulation of BDNF expression. However, the presence of the PROG membrane-binding protein 25-Dx [20], which increases after PROG administration to rats with SCI, suggests this molecule may become important for PROG effects under pathological conditions such as SCI. Also, the conversion of PROG to its metabolites 5α-dihydroprogesterone and 3α,5α-tetrahydroprogesterone [23], may be affecting BDNF expression. These reduced derivatives modulate inhibitory and excitatory neurotransmission at the cell membrane [24]. Since interplay between neurotransmitter receptor systems can regulate BDNF expression [43,44], PROG effects may be driven through these intermediates. These demonstrations support that PROG effects are pleiotropic and can be achieved via different mechanisms involving different receptors [45].

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Table 1
Similarities in cellular effects of progesterone and BNDF

Molecule or cellular event changed	Progesterone	BDNF	References
Choline acetyl-transferase	⇑	⇑	[13,25,26]
Na,K-ATPase	⇑	⇑	[13,45]
GAP-43	⇑	⇑	[13,27]
Myelin basic protein	⇑	⇑	[18,29]
Chromatolysis, neuronal degeneration	⇓	⇓	[32,46]

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