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Modulation of NADPH-diaphorase and glial fibrillary acidic protein by progesterone in astrocytes from normal and injured rat spinal cord

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Abstract

Progesterone (P4) can be synthesized in both central and peripheral nervous system (PNS) and exerts trophic effects in the PNS. To study its potential effects in the spinal cord, we investigated P4 modulation (4 mg/kg/day for 3 days) of two proteins responding to injury: NADPH-diaphorase, an enzyme with nitric oxide synthase activity, and glial fibrillary acidic protein (GFAP), a marker of astrocyte reactivity. The proteins were studied at three levels of the spinal cord from rats with total transection (TRX) at T10: above (T5 level), below (L1 level) and caudal to the lesion (L3 level). Equivalent regions were dissected in controls. The number and area of NADPH-diaphorase active or GFAP immunoreactive astrocytes/0.1 mm^2 in white matter (lateral funiculus) or gray matter (Lamina IX) was measured by computerized image analysis. In controls, P4 increased the number of GFAP-immunoreactive astrocytes in gray and white matter at all levels of the spinal cord, while astrocyte area also increased in white matter throughout and in gray matter at the T5 region. In control rats P4 did not change NADPH-diaphorase activity. In rats with TRX and not receiving hormone, a general up-regulation of the number and area of GFAP-positive astrocytes was found at all levels of the spinal cord. In rats with TRX, P4 did not change the already high GFAP-expression. In the TRX group, instead, P4 increased the number and area of NADPH-diaphorase active astrocytes in white and gray matter immediately above and below, but not caudal to the lesion. Thus, the response of the two proteins to P4 was conditioned by environmental factors, in that NADPH-diaphorase activity was hormonally modulated in astrocytes reacting to trauma, whereas up-regulation of GFAP by P4 was produced in resting astrocytes from non-injured animals.

Keywords: Astrocytes; Injury; NADPH-diaphorase; Progesterone; Spinal cord

1. Introduction

Steroid hormones secreted by the adrenal glands and gonads, or those synthesized within the nervous system (''neurosteroids'') may provide trophic support to neurons and glial cells in experimental and clinical cases of CNS injury [1–3]. The efficacy of glucocorticoids and their derivatized 21-aminosteroids was early demonstrated in patients suffering from spinal cord trauma [4]. It has been proposed that glucocorticoid neuroprotection relies mainly on anti-inflammatory properties exerted upon activated microglia/macrophages [5]. However, glucocorticoids can also modulate neuronal parameters related to regeneration in animals with injured spinal cord [6,7].

Besides glucocorticoids, gonadal hormones and neu-

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rosteroids synthesized by glial cells and some populations of neurons [8–10] play an important role in neuronal and glial cell plasticity during regeneration [3,11]. Particularly interesting is the observation that pregnenolone, a direct precursor of progesterone (P4), facilitates recovery after spinal cord injury in rats [12]. P4, in turn, prevented the neuron loss in the hypoglossal and facial motor nuclei damaged by lesion cuts [13]. P4 can be synthesized by myelinating glial cells, which express an intracellular receptor for this hormone [11,14]. In these cells, the hormone activates the promoters of genes encoding myelin proteins [11,15]. Thus, the production of P4 appears a feature of myelinating glial cells and neurons [8,10]. Interestingly, in the regenerating sciatic nerve of male mice, P4 synthesized by Schwann cells promotes the formation of new myelin sheaths after lesion [16].

In contrast to observations in myelin-producing cells and in several regions of the CNS, demonstration of a classical receptor or non-genomic mechanisms of action for P4 in the spinal cord are still lacking. The spinal cord may respond to blood-derived hormone and to locally synthesized P4, since it contains the enzymatic machinery for its synthesis [17] (Guennoun et al., unpublished results). Whatever the molecular mechanisms used, some P4 functions in the spinal cord have been reported. These include effects related to nociception, such as modulation of dynorphin levels in the lumbar region, enhancement of antinociceptive effects of muscimol and coincident activation of kappa and delta opiate receptors [18–20]. Additionally, levels of substance P, neurokinin A and calcitonin gene-related peptide and kainate-induced currents are regulated by P4 in the spinal cord [21–23].

We have now investigated the effects of P4 in rats with spinal cord transection in the same model successfully used before to study glucocorticoid effects in rats with spinal cord injury [6,7]. Two proteins draw our attention: the glial fibrillary acidic protein (GFAP) and the NADPH-diaphorase. Induction of GFAP, an astrocyte intermediate filament cytoskeletal protein, is considered the main indicator of astroglial activation caused by CNS injury [24]. Steroid hormones, among them P4, cytokines and other factors regulate expression of GFAP in brain and astrocyte culture under normal and pathological conditions [25,28]. NADPH-diaphorase has been considered a histochemical marker for nitric oxide synthase (NOS). It has a restricted distribution in normal neurons but is found in resting astrocytes [29,30]. Oligodendrocytes are devoid of nitric oxide synthase [31]. Reports demonstrated that injury, ischemia and cytokines stimulate astroglial NOS [32,34]. Estrogens, gluco and mineralocorticoids also modulate this enzyme activity in neurons [35–37]. Considering (1) the postulated roles of nitric oxide (NO) not only in cytotoxicity but also in enhancement of cell function [32,36,38], (2) the importance of GFAP for astrocyte activation and for myelin repair and deposition [24,39], we studied if P4 regulates the expression of these proteins in astrocytes from rats with spinal cord transection. Our data demonstrated that NADPH-diaphorase activity was hormonally modulated in astrocytes reacting to trauma, whereas regulation of GFAP immunoreactivity by P4 was mainly produced in resting but not reactive astrocytes.

2. Materials and methods

2.1. Materials

Progesterone (P4, Proluton) was a kind gift of Schering Laboratories (Argentina). Goat serum and the Vectastain ABC Elite Kit were purchased from Vector Labs. (Burlingame, CA, USA). Diaminobenzidine tetrahydrochloride, nitroblue tetrazolium, NADPH, malic acid and GFAP antiserum (rabbit polyclonal antiserum cat. G9269) were purchased from Sigma Chem. Co. (St. Louis, MO, USA.). Paraformaldehyde was obtained from Riedel-de Haen AG (Seeize, Germany) and Permount from Fischer Scientific (Pittsburgh, PA, USA). All other chemicals were of the highest purity commercially available.

2.2. Experimental animals

Male Sprague–Dawley rats (250–300 g) were deeply anesthetized with ketamine (60 mg/kg, i.p.) and divided into four groups: Groups 1 and 2 were sham-operated, in which case soft tissues were cut and then sutured. In Groups 3 and 4, laminectomy was performed and spinal cord transection (TRX) was carried out at thoracic level T10 using the sharp edge of a 25G needle [6,7]. Groups 1 and 3 received vehicle injections, whereas animals in Groups 2 and 4 received four injections of 4 mg/kg P4 at times 1 h (i.p.), and again at 24, 48 and 72 h (s.c.) post-lesion. This dose of P4 was chosen because it prevented secondary neuronal degeneration and loss after brain injury in rats [40]. Thus, Groups 1–4 were labeled control, control + P4, TRX and TRX + P4, respectively. All vehicle and progestin-treated animals were used for the different experiments exactly 75 h after sham surgery or TRX, and 3 h after receiving the last injection. Regions analyzed for NADPH-diaphorase activity and GFAP immunocytochemistry after spinal cord TRX at T10 included above (T5 level), below (L1 level) and caudal to the lesion (L3 level). Equivalent regions were dissected from the spinal cord of sham-operated rats. The studies were carried out according to international guidelines on the care and use of experimental animals.

Efforts were made to keep the number of animals with TRX at a minimum.

2.3. NADPH-diaphorase histochemistry

After rats were fully anesthetized with ether, animals were heart-perfused with 50 ml 0.9% (w/v) NaCl, followed by 50 ml 2% (w/v) paraformaldehyde. Spinal cords from the four groups described above ($n = 4$ animals per group) were removed, placed in 2% (w/v) paraformaldehyde in 0.1 M phosphate buffer pH 7.2 for 2 h and then cryoprotected in 30% (w/v) sucrose for 24 h. A slight modification of the method of Vincent and Kimura [41] was employed to determine NADPH-diaphorase activity [42]. Cryostat sections of spinal cord regions (16 μm) were fixed by immersion in 2% (w/v) paraformaldehyde in 0.1 M phosphate buffer pH 7.2 during 6 min at 4°C. After fixation, the sections were rinsed twice in PBS, and incubated in a solution of 0.1 M Tris–HCl buffer pH 7.4 containing 0.3% (v/v) Triton X-100, 0.2 mg/ml of nitroblue tetrazolium, 2.7 mg/ml l-malic acid, and 1 mg/ml of β-NADPH. After keeping the reaction in the darkness during 60 min at 37°C, it was stopped by two washes in PBS at room temperature. Sections were then dehydrated briefly in ethanol, dried and coverslipped with Permount.

2.4. GFAP immunocytochemistry

Ether anesthetized rats from the four groups described above ($n = 4$ animals per group) were perfused with 0.9% (w/v) cold NaCl through the heart and spinal cords removed and dissected as specified above. Sections (above, below and caudal to the lesion) were fixed in 10% (v/v) formalin for 48 h and embedded in paraffin. Immunocytochemistry was performed as previously described [43]. Five μm sections were washed in PBS, preincubated in 10% (v/v) goat serum, and incubated overnight at 4°C with a 1/250 dilution of anti-GFAP serum in a moist atmosphere. Following a wash with PBS, the primary antibody bound to the sections was revealed using a biotinylated goat anti-rabbit complex. For the final reaction products, sections were exposed to 0.25 mg/ml diaminobenzidine tetrachloride and 0.01% (v/v) H_2O_2 for 6–8 min in the dark. The sections were then given a rinse in PBS, dehydrated in graded ethanols and xylene, and mounted with Permount.

2.5. Quantitative analysis

Astrocytes were observed with an Olympus optic microscope equipped with a videocamera. Regions selected for quantitation included the lateral funiculus of the white matter and on Lamina IX of the gray matter, with measurements carried out in at least 10 sections per animal per spinal cord area located above (T5 level), below (L1 level) and caudal to the lesion (L3 level). Using the Optimas Program [44], we determined the total number of astrocytes and area of each cell with increased immunochemical (GFAP) or histochemical signal (NADPH-diaphorase). Results were normalized to 0.1 mm^2 of gray and white matter [42]. Because experimental treatments produced size heterogeneity of spinal cord astrocytes, we also quantified the number of NADPH-diaphorase positive and GFAP immunoreactive astrocytes equal or higher than 70 $\mu m^2/0.1$ mm^2 for the white matter, and equal or higher than 55 $\mu m^2/0.1$ mm^2 for the gray matter.

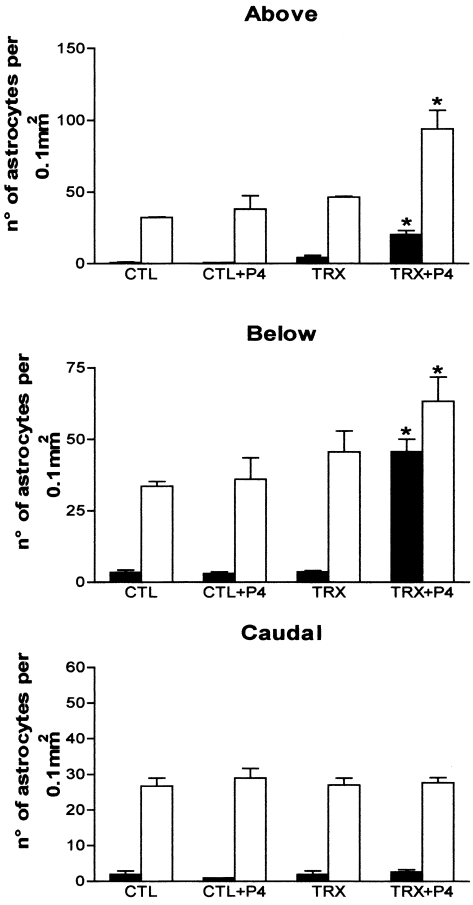

Fig. 1. Progesterone (P4) effects on the total number of NADPH-diaphorase positive astrocytes of normal and transected spinal cords. Data correspond to astrocyte number per 0.1 mm^2 in the white matter (lateral funiculus, white bars) and gray matter (Lamina IX, dark bars). Group labeling: CTL, control sham-operated rats; CTL + P4, controls receiving P4; TRX, spinal cord transection; TRX + P4, transection plus P4. Cells were counted above (T5 level, upper graph), below (L1 level, middle graph) or caudal (L3 level, lower graph) to the lesion placed at T10. Results represent the mean ± SEM of $n = 4$ animals per group. Statistical significance was carried out by ANOVA and post-hoc tests. For white and gray matter astrocytes located above and below the lesion: ∗TRX + P4: $p < 0.05$ vs. all other groups.

While small cells correspond mainly to bipolar or radial astrocytes, large stellate forms are typical of reactive astrocytes [45]. Photography was carried out with a Zeiss Axioplan optic microscope equipped with an automatic OM camera.

2.6. Statistical analysis

All data are presented as mean ± SEM. Results were analyzed by one way ANOVA followed by post-hoc Newman-Keuls test. Statistical significance was established at $p < 0.05$.

3. Results

3.1. NADPH-diaphorase histochemistry

Data of NADPH-diaphorase histochemistry in the four groups of animals (control, control + P4, TRX and TRX + P4) are presented in Figs. 1 and 2. In control animals, P4 had no effect on the total number of NADPH-diaphorase positive astrocytes at all levels of the spinal cord analyzed (Fig. 1). In animals with TRX and not receiving hormone, the number of astrocytes remained similar to the controls and controls + P4. In the TRX group, instead, P4 increased the total number of NADPH-diaphorase active astrocytes in white and gray matter immediately above and below, but not caudal to the lesion ($p < 0.05$ or less, Fig. 1).

The area of NADPH-diaphorase positive astrocytes was also differentially regulated by P4, resembling in part data on astrocyte number. In the control group, P4 did not change the area of NADPH-diaphorase active astrocytes (Fig. 2). In contrast to data on astrocyte number, in the TRX group there was a significant up-regulation of the histochemically identified astrocyte area in white matter above the lesion and in gray matter caudal to trauma with respect to the sham-operated control group ($p < 0.05$ versus. controls and controls + P4). In the group with combined treatment (TRX + P4) the area of astrocytes stained for NADPH-diaphorase was increased in the gray matter above and below the lesion ($p < 0.05$ or less versus the other groups), without effect in the caudal region. Clearly, modulation by P4 of NADPH-diaphorase was predominantly observed in astrocytes sensitized by the stimulus of spinal cord lesion.

In Table 1 the number of large NADPH-diaphorase positive astrocytes in gray and white matter located above, below and caudal to the lesion is presented. These data demonstrated that large size NADPH-diaphorase positive astrocytes measuring >55 μm^2 in the gray matter and >70 μm^2 in the white matter, were more frequent in the TRX + P4 group than in the other groups both above and below the lesion. Fig. 3 shows the morphological changes of NADPH-diaphorase positive astrocytes from the white matter (left panels) and gray matter (right panels) located below the lesion in controls, controls + P4, TRX, and TRX + P4.

3.2. GFAP immunocytochemistry

Data on GFAP immunostaining in the four group of animals are presented in Figs. 4 and 5. In control rats, P4 produced a moderate, still significant up-regulation of the total number of GFAP-expressing astrocytes in gray and white matter at all levels of the spinal cord (control + P4: $p < 0.05$ or less versus. control group; Fig. 4). Spinal cord TRX per se was also a powerful stimulus for GFAP expression in gray and white mater distribu-

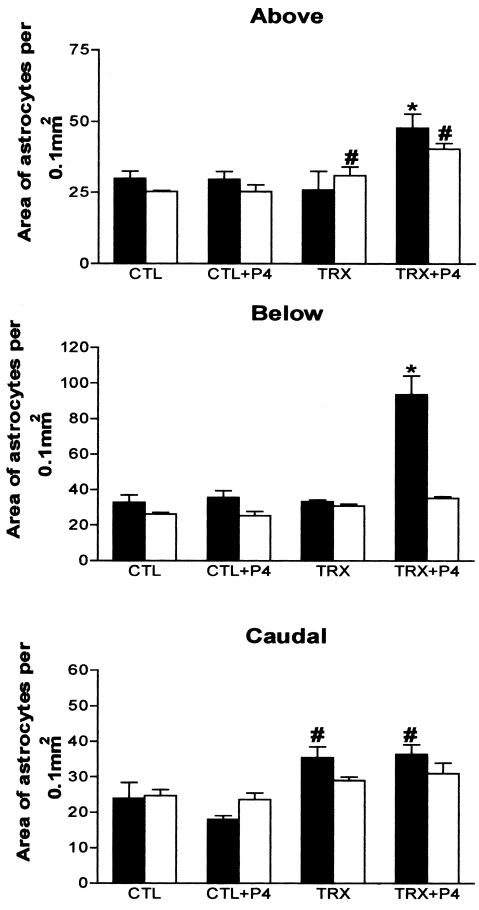

Fig. 2. Effects of progesterone (P4) and spinal cord transection on the area of NADPH-diaphorase positive astrocytes per 0.1 mm^2 in the white matter (lateral funiculus, white bars) and gray matter (Lamina IX, dark bars). Group labeling as in the legend to Fig. 1. Significance: for gray matter astrocytes located above and below the lesion: ∗TRX + P4: $p < 0.05$ vs. all other groups, whereas for white matter astrocytes resident above and for gray matter astrocytes caudal to the lesion #TRX and TRX + P4: $p < 0.05$ vs. CTL.

ted above, below and caudal to the lesion (TRX $p < 0.05$ or less versus. sham-operated controls). After TRX, P4 treatment did not modify the already large number of GFAP immunoreactive astrocytes. The exception to this finding was a TRX + P4 effect in the gray matter astrocytes caudal to the lesion ($p < 0.05$ vs. TRX alone).

In control animals, P4 increased the GFAP immunoreactive area of white matter astrocytes located above, below and caudal to the lesion as well as in gray matter astrocytes at the T5 level ($p < 0.05$ versus. untreated control group; Fig. 5). As shown above for astrocyte number, spinal cord TRX significantly increased the cell area showing GFAP expression in white and gray matter ascribed to all regions of the spinal cord ($p < 0.05$ or less versus. the sham-operated control group). In agreement with findings on astrocyte number, P4 was unable to modify the large GFAP immunoreactive area of astrocytes following spinal cord transection (TRX vs. TRX + P4: non-significant). Thus, while TRX was a powerful stimulus for GFAP expression, P4 effects on this protein required quiescent astrocytes, as shown in control rats.

Astrocyte morphology, revealed by GFAP immunostaining, was dramatically changed by the lesion and P4 treatment. Data in Table 2 lists the number of large astrocytes in the four groups of animals. Thus, large stellate GFAP-positive astrocytes predominated in white matter (>70 μm^2) and gray matter (>55 μm^2) in control + P4, TRX and TRX + P4 groups over the sham-operated control animals. Finally, Fig. 6 shows photomicrographs of astrocytes in the white matter (left panel) and gray matter (right panel) above the lesion in controls, controls + P4, TRX and TRX + P4 groups. Clearly, predominance of large, stellate GFAP-positive astrocytes characterized the last three groups.

4. Discussion

This work is the first report showing P4 effects on astroglial cells of the spinal cord. Since P4 actions on Schwann cells were already reported [3,16], both central and peripheral glial cells may be potential targets of the progestin. In agreement with Morin-Richaud et al. [46], total spinal cord TRX constitutes a good model to study glial cell responses to injury. These authors showed that peak astrocyte activation occurs 3 days after injury, which is within the time framework employed by us to analyze P4 effects. In previous work, we demonstrated that 3 days following transection, glucocorticoids modulated some neuronal and glial cell parameters in the rat spinal cord [6,7].

To investigate possible functions of P4 in the lesioned spinal cord, a dose known to prevent secondary neuronal degeneration following brain injury was chosen [40]. We analyzed the behavior of two astrocyte proteins responding to injury, namely NADPH-diaphorase and GFAP. Results showed that P4 increased the number of astrocytes positive for NADPH-diaphorase in the injured spinal cord immediately above and below, but not caudal to the lesion. This change comprised both fibrous (white matter) and protoplasmic astrocytes (gray matter). Rats with spinal cord TRX but free of P4 treatment, also up-regulated enzyme activity in white matter above, and in gray matter caudal to the lesion. However, P4 increased this response to injury. Because P4 was ineffective in control animals, it is likely that the response of NADPH-diaphorase to P4 was potentiated by environmental factors developing during trauma. Based on current evidence, it is possible that factors such as cytokines, growth factors or glutamate released during injury [34,38,47] conditioned the sensitivity to P4 of reactive astrocytes. Because these factors affected proximal astrocytes (i.e., above and below the lesion site), they

Table 1
Number of large NADPH-diaphorase positive astrocytes resident in the gray and white matter located above, below and caudal to the lesion placed at T10. Group labeling: CTL, control sham-operated rats; CTL + P4, controls receiving P4; TRX, spinal cord transection and TRX + P4, transection plus P4 groups

Number of large NADPH-diaphorase positive astrocytes/0.1 mm^2

	Above		Below		Caudal	
	Gray matter (>55 μm^2)	White matter (>70 μm^2)	Gray matter (>55 μm^2)	White matter (>70 μm^2)	Gray matter (>55 μm^2)	White matter (>70 μm^2)
CTL	0 ± 0	1.2 ± 1.0	0 ± 0	1.2 ± 1.0	0 ± 0	0.6 ± 0.3
CTL + P4	0 ± 0	0.7 ± 0.5	0 ± 0	1.0 ± 0.8	0 ± 0	0.5 ± 0.1
TRX	0.1 ± 0.01	4.3 ± 0.4	0 ± 0	2.5 ± 0.5	0 ± 0	1.6 ± 0.5
TRX + P4	5.0 ± 3.2[a]	15 ± 3.5[b]	25.7 ± 7.3[b]	7.3 ± 2.4[a]	0 ± 0	1.7 ± 1.3

[a] $p < 0.05$.
[b] $p < 0.001$ vs. CTL, CTL + P4 and TRX groups in the same column.

may be acting locally without diffusion to the caudal region of the spinal cord.

Progestin treatment also produced morphological changes in spinal cord astrocytes, in that NADPH-diaphorase positive astrocytes changed from predominantly flat to a stellate profile. The proportion of large astrocytes was also significantly increased by P4 in gray and white matter. Changes in morphology are not unique to the P4 effects in the spinal cord, however, as treatment with estradiol resulted in astrocyte differentiation from epithelioid to radial and stellated shapes in hypothalamic cultures [48]. These data, as well as ours, suggest that the sex hormones estrogen and progesterone may regulate astrocyte differentiation. Sex steroids also regulate NADPH-diaphorase in other regions of the CNS. For example, NADPH-diaphorase activity and NOS transcription showed positive regulation by estradiol in preoptic area neurons [35] and endothelium [49]. In contrast to sex steroids, negative regulation of NOS/NADPH-diaphorase activity was caused by natural or synthetic adrenal steroids in brain neurons and endothelium [36,37,50] and in spinal cord motoneurons [42]. Thus, several steroid hormones regulate this enzyme activity and/or

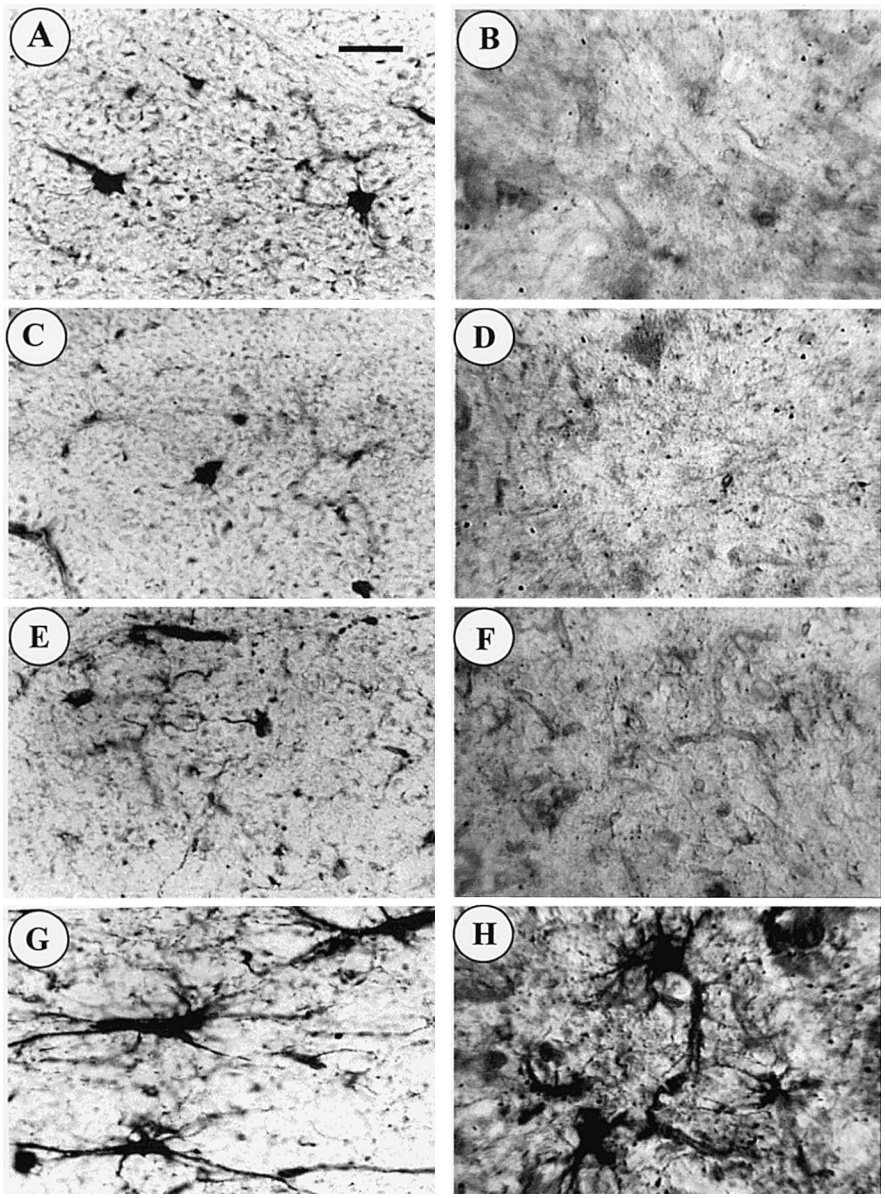

Fig. 3. Representative NADPH-diaphorase staining of astrocytes from the white matter (left panels) and gray matter (right panels) localized below the lesion at T10 or a similar region taken from sham-operated rats. Group labeling as in the legend to Fig. 1. The photomicrographs represent CTL (A, B), CTL + P4 (C,D), TRX (E,F) and TRX + P4 (G, H) groups. Staining of large, stellate astrocytic forms predominate in (G, H). Magnification 1000 ×, no counterstaining. Scale bar 50 μm.

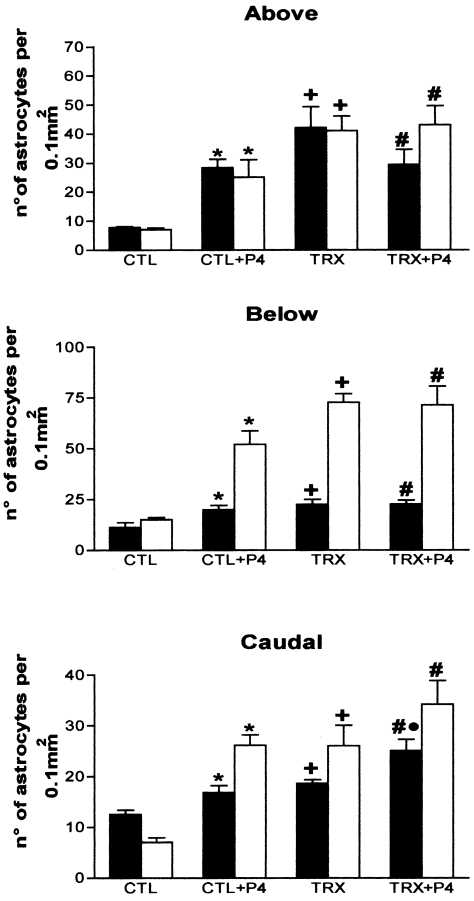

Fig. 4. Progesterone (P4) effects on the total number of immunoreactive GFAP astrocytes of normal and transected spinal cords. Data correspond to astrocyte number per 0.1 mm^2 in the white matter (white bars) and gray matter (dark bars). Group labeling as in the legend to Fig. 1. Significance: for white and gray matter astrocytes located above, below and caudal to the lesion: ∗CTL + P4: $p < 0.05$ vs. CTL; $^{+}$TRX and $^{\#}$TRX + P4: $p < 0.05$ vs. CTL. However, TRX vs. TRX + P4: NS. For gray matter astrocytes of the caudal region: • TRX + P4: $p < 0.05$ vs. all other groups.

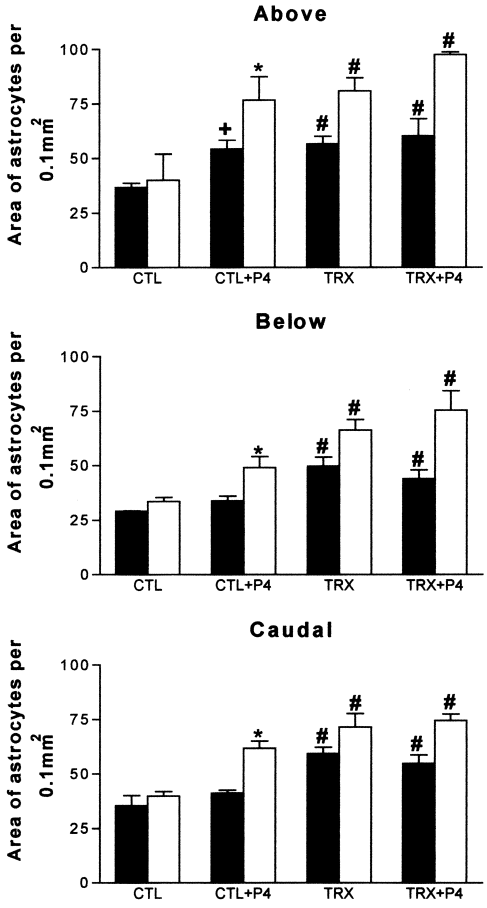

Fig. 5. Effects of progesterone (P4) and spinal cord transection on the area of GFAP-immunoreactive astrocytes per 0.1 mm^2in the white matter (lateral funiculus, white bars) and gray matter (Lamina IX, dark bars). Group labeling as in the legend to Fig. 1. Significance: for white matter astrocytes located above, below and caudal to the lesion: ∗CTL + P4: $p < 0.05$ vs CTL. For gray matter astrocytes above the lesion: $^{+}$CTL + P4: $p < 0.05$ vs. CTL. For white and gray matter astrocytes located in the three regions: $^{\#}$TRX or TRX + P4: $p < 0.05$ or less versus. CTL. However, TRX + P4 had no additional effects in any region respect of TRX alone.

Table 2
Number of large GFAP-immunoreactive astrocytes resident in the gray and white matter located above, below and caudal to the lesion placed at T10. Group labeling: CTL, control sham-operated rats; CTL + P4, controls receiving P4; TRX, spinal cord transection and TRX + P4, transection plus P4 groups

Number of large GFAP-immunoreactive astrocytes/0.1 mm^2						
	Above		Below		Caudal	
	Gray matter (> 55 μm^2)	White matter (> 70 μm^2)	Gray matter (> 55 μm^2)	White matter (> 70 μm^2)	Gray matter (> 55 μm^2)	White matter (> 70 μm^2)
CTL	0.3 ± 0.23	0.12 ± 0.2	0.32 ± 0.1	1.1 ± 0.7	0.25 ± 0.4	0.1 ± 0.07
CTL + P4	9.3 ± 0.5^{b}	13.2 ± 1.7^{a}	2.5 ± 1.2^{a}	8.3 ± 3.1^{a}	4.2 ± 1.3^{a}	7.8 ± 1.64^{b}
TRX	12.6 ± 3.1^{c}	14.9 ± 3.4^{b}	9.2 ± 3.1^{a}	20.7 ± 4.2^{b}	7.6 ± 0.3^{b}	8.33 ± 1.5^{b}
TRX + P4	12.9 ± 3.6^{c}	18.6 ± 3.7^{c}	6.7 ± 2.9^{a}	15.8 ± 2.6^{b}	10.3 ± 2.7^{c}	12.3 ± 3.1^{c}

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$ vs. CTL group in the same column.

expression, although the direction of the change may depend on the hormone in question and the normal or pathological state of the tissue.

NADPH-diaphorase staining, however, did not indicate which enzyme isoform was the target of P4 effects. Astrocytes can express all known NOS isoforms. First, the calcium-independent inducible form (iNOS) was found in cytokine-stimulated astrocytes, responding to inflammation and neurodegeneration [47]. Second and third, the constitutive calcium/calmodulin dependent isoforms eNOS (endothelial) and nNOS (neuronal) were also described in astrocytes from injured areas [30,38]. Increased NO generation may be toxic or cytoprotective, with literature reports supporting both alternatives. Thus, stimulated astrocytes may be neurotoxic via a NO-mediated mechanism [33]. NO is involved in several neuropathologies, including spinal cord cavitation [51,52]. Excess endothelial-derived NO is pro-inflammatory, an effect inhibited by glucocorticoids [50]. In contrast, other authors considered that early NOS expression by neurons is associated to axonal sprouting and growth in the spinal cord [29] and increased NO may be beneficial to neuronal function after a region has been damaged [32]. In this respect,

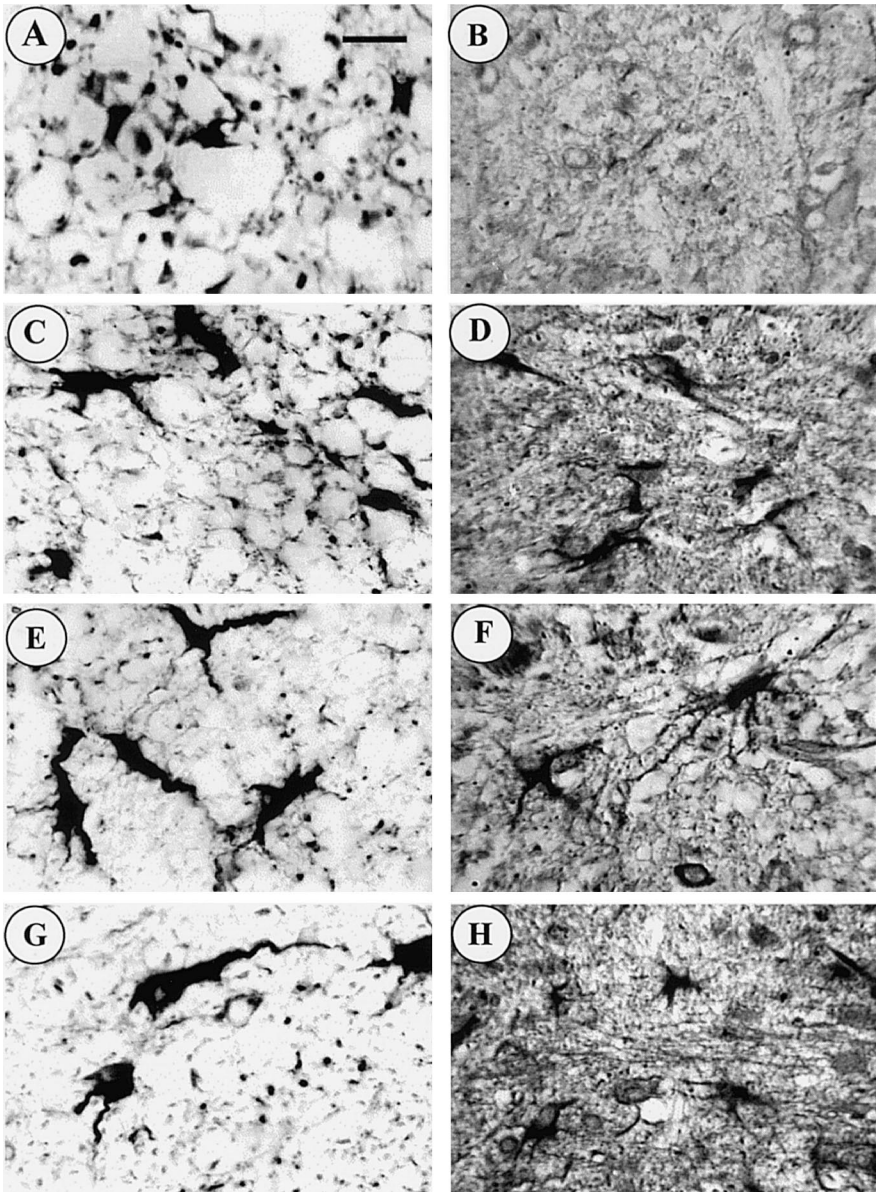

Fig. 6. Representative GFAP immunostaining of astrocytes from the white matter (left panels) and the gray matter (right panel) located below the lesion at T10 or a similar region taken from sham-operated rats. Photomicrographs represent control (A, B), control + P4 (C, D), TRX (E, F) and TRX + P4 (G, H) groups. Large, stellate astrocytes showing GFAP immunostaining characterized groups B–H in comparison to small astrocytes found in A and B. Magnification 1000 × , no counterstaining. Scale bar 50 μm.

local release of NO could block NMDA receptors, attenuating lesion-induced excitotoxicity [38]. However, in spite of the fact that NADPH-diaphorase may be a histochemical marker for NOS, discrepancies exist regarding their identity [53]. In preliminary experiments for immunocytochemical detection of NOS using isoform-specific antibodies, abundant nNOS positive astrocytes were observed in the spinal cord from rats with TRX receiving P4. In these experiments, astrocytes remained iNOS negative, whereas LPS-stimulated peritoneal macrophages strongly reacted with the iNOS antibody (Labombarda et al., unpublished).

Effects of P4 on GFAP protein expression, instead, differ from those on NADPH-diaphorase activity. Thus, P4 increased the number and area of GFAP-expressing astrocytes resident in the gray and white matter at all levels of the spinal cord from sham-operated rats, whereas it was ineffective in rats with TRX with the exception of the gray matter in the caudal region. This finding suggests that up-regulation of GFAP by P4 was effected in resting astrocytes distributed throughout the spinal cord, perhaps responding directly to exogenous P4. The fact that P4 effects on NADPH-diaphorase were anatomically restricted to regions above and below the lesion only, further supports that stimulation of this enzyme occurred in activated proximal astrocytes only.

Furthermore, GFAP-positive astrocytes from P4-treated animals, as well as those from rats with TRX, presented long processes and a stellated morphology, with reduced number of flat, epitheloid forms. A similar change was described in hippocampal cultured astrocytes added with estradiol, testosterone, pregnenolone and dehidroepiandrosterone [54]. In these circumstances, astrocytes were transformed into hypertrophic, highly GFAP immunoreactive cells in magnitude dependent on the hormonal treatment. The stimulatory effect of estradiol on GFAP may involve genomic mechanisms [27,28]. In contrast to the stimulatory effects of estrogens and neurosteroids on GFAP-expressing astrocytes, glucocorticoids down-regulate GFAP mRNA and protein expression probably by receptor interaction with hormone-responsive elements (HRE) in the 5′-upstream promoter [25,26]. Although GRE/P_4RE [55] have been identified in the promoter region of the GFAP gene, a direct interaction with the P4 receptor has so far not been demonstrated.

One other report considered P4 effects on GFAP-immunolabeled reactive astrocytes after CNS injury. After a penetrating brain injury, decreased proliferation of GFAP-immunoreactive astrocytes in the vicinity of the wound was obtained in P4-treated rats [56]. In our experiments, P4 administered in vivo enhanced the number and area of GFAP-labeled astrocytes from non-wounded animals, while it was inactive in the transected group. However, brain and spinal cord astrocytes may respond differently to injury, because pronounced astrocyte heterogeneity exists in the CNS [45]. Besides, after spinal cord lesion the GFAP gene may be maximally stimulated and unresponsive to hormonal treatment. Along this line, Laping et al. [26] considered that local injury takes precedence over hormone-mediated responses at the GFAP gene. This conclusion, however, needs experimental demonstration of a genomic, receptor-mediated mechanism of action in the spinal cord. Although proof for a classical P4 receptor in the spinal cord is not available, preliminary observations using the KC 146 monoclonal antibody [57] demonstrated the specific staining of neurons and glial cells (unpublished results). However, the level at which P4 acts on astrocytes still remains unclear and the possibility of indirect effects should also be considered. In this sense, convergence of progestin with growth factors (i.e., epidermal growth factor, basic fibroblast growth factor, insulin-like growth factors I and II and their receptors) has been demonstrated in reproductive tissues [58–60].

The induction of a reactive response in quiescent astrocytes by P4 treatment may have functional consequences. Although traditionally astrocytosis was considered a negative factor for neuronal trophism and regeneration [34,61], recent data support that reactive astrocytes bring neuroprotection. Thus, astrocytes may help neuronal regeneration and stop neuronal death by secretion of growth factors, substrate-bound neurite promoting factors and removal of neurotoxins and glutamate [24,62,63]. GFAP-expressing astrocytes may be also necessary for myelination and normal white matter architecture [39]. If true for the spinal cord, P4 might help myelin repair by a dual mechanism: firstly, by activating the promoters of genes coding for myelin proteins in oligodendrocytes, as demonstrated previously for Schwann cells [15,64]; secondly, by increasing GFAP expression in non-injured astrocytes.

In conclusion, we showed that the response of two astrocyte proteins to P4 was conditioned by environmental factors, in that NADPH-diaphorase activity was hormonally modulated in astrocytes reacting to trauma, whereas up-regulation of GFAP by P4 was effected in resting astrocytes. However, it remains elusive why both parameters respond differently and whether this response should be considered as a neuroprotective action or a negative factor. It would be necessary to assess neuronal parameters, the response of other glial cell types and some functional measurements in order to interpret the pathophysiological significance of P4 effects in animals with injured spinal cord.

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