ELSEVIER

Brain Research 912 (2001) 144–153

BRAIN RESEARCH

www.elsevier.com/locate/bres

Research report

Glucocorticoid effects on Fos immunoreactivity and NADPH-diaphorase histochemical staining following spinal cord injury

Susana González[a,b], Florencia Labombarda[a,b], María Claudia Gonzalez Deniselle[a,b], Flavia E. Saravia[a,b], Paulina Roig[a], Alejandro F. De Nicola[a,b,c,*]

[a]*Laboratory of Neuroendocrine Biochemistry, Instituto de Biología y Medicina Experimental, Obligado 2490, 1428 Buenos Aires, Argentina*
[b]*Department of Human Biochemistry, Faculty of Medicine, University of Buenos Aires, Buenos Aires, Argentina*
[c]*Instituto Universitario de Ciencias de la Salud, Fundación Barceló, Buenos Aires, Argentina*

Accepted 15 May 2001

Abstract

Glucocorticoids (GC) provide neuroprotection and early recovery after spinal cord injury (SCI). While several mechanisms were proposed to account for these effects, limited information exists regarding GC actions in sensory areas of the spinal cord. Presently, we studied the time course of Fos expression, and reduced nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemical staining to monitor neuronal responses to SCI with or without GC treatment. Rats with sham-operation or transection at the thoracic level (T_7–T_8) received vehicle or 5 mg/kg of the GC dexamethasone (DEX) at 5 min post-lesion and were sacrificed 2 or 4 h after surgery. Another group of SCI rats received vehicle or intensive DEX treatment (5 min, 6 h, 18 h and 46 h post-lesion) and were sacrificed 48 h after surgery. The number of NADPH-d positive neurons or Fos immunoreactive nuclei was studied by computer-assisted image analysis in superficial dorsal horn (Laminae I–III) and central canal area (Lamina X) below the lesion. While constitutive Fos immunoreactive nuclei were sparse in controls, SCI increased Fos expression at 2 and 4 h after injury. DEX treatment significantly enhanced the number of Fos positive nuclei in Laminae I–III by 4 h after transection, although the response was not maintained by intensive steroid treatment when tested at 48 h after SCI. NADPH-d positive neurons in Laminae I–III increased at 2 and 4 h after SCI while a delayed increased was found in central canal area (Lamina X). DEX treatment decreased NADPH-d positive neurons to sham-operated levels at all time points examined. Thus, while GC stimulation of Fos suggests activation of neurons involved in sympathetic outflow and/or pain, down-regulation of NADPH-d indicates attenuation of nociceptive outflow, considering the role of enzyme-derived nitric oxide in pain-related mechanisms. Differential hormonal effects on these molecules agree with their localization in different cell populations. © 2001 Elsevier Science B.V. All rights reserved.

Theme: Endocrine and autonomic regulation

Topic: Neuroendocrine regulation: other

Keywords: Glucocorticoid; Spinal cord injury; Dexamethasone; Fos; NADPH–diaphorase; Neuroprotection

1. Introduction

Glucocorticoids (GC) enhance functional recovery and induce trophic and regenerative responses after spinal cord injury (SCI) in humans and experimental animals [1,3,9,11,27,46]. Success of early hormonal therapy was partly attributed to GC antiinflammatory activity [1,68], although treatment with GC in megadoses and with anti-oxidant 21-aminosteroids also provide neuroprotection by antagonizing free-radical and lipid peroxide damage caused by SCI [5,27,28].

GC receptors (GR) were described in different neuronal populations of the spinal cord [10,13,15,18,37]. Interestingly, SCI increased neuronal GR, as demonstrated by binding assays, immunocytochemistry and Western blot [19,65]. In motoneurons from rats with SCI, acute GC treatment increased the activity and biosynthesis of Na,K-ATPase subunit mRNA [5,22], restored choline-acetyl-transferase expression [23], modulated immunoreactivity for the low affinity neurotrophin receptor [4,23] and

*Corresponding author. Tel.: +54-11-4783-2869; fax: +54-11-4786-2564.
E-mail address: denicola@proteus.dna.uba.ar (A.F. De Nicola).

0006-8993/01/$ – see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0006-8993(01)02717-2

stimulated ornithine decarboxylase activity and polyamine levels [21]. GC also decreased neurofilament degradation and restored neuronal energy metabolism [28]. In the long term, GC stimulated axonal regeneration, sprouting and neuronal survival of rats with SCI [9,46]. In contrast, less information is available about GC effects in sensory areas of the spinal cord, although GR were also described in dorsal horn neurons [10]. In sensory areas, GC effects include enhancement of glutamate binding to *N*-methyl-D-aspartate (NMDA) receptors [21] and reduction of the inhibitory benzodiazepine receptor system [20].

In the present study, we examined the effects of GC in the superficial dorsal horn (Laminae I–III) and central canal region (Lamina X), areas involved in the transmission of nociceptive inputs and control of sympathetic outflow [17,26]. The protein product of the proto-oncogene c-fos, Fos, is considered a marker of neuronal activation which increases after SCI in dorsal horn and central canal [29,31,52]. A high percentage of Fos labeled nuclei of the dorsal horn colocalizes with preproenkephalin and preprodynorphin, suggesting Fos participation in nociception [12]. However, Ruggiero et al. [52] considered that neurons expressing Fos after SCI are interneurons regulating sympathetic outflow. Previous reports on GC interaction with Fos in the hypothalamus and spinal cord [6,7,36] as well as evidence for the colocalization of Fos with GR in the dorsal horn [10] encouraged us to study this parameter.

Another example of gene switching in response to neuronal activation in the sensory spinal cord areas is the expression of nitric oxide synthase (NOS), an enzyme exhibiting adenine dinucleotide phosphate-diaphorase (NADPH-d) activity [44,66]. NO may be formed postsynaptically in response to NMDA receptor activation [17], and some reports revealed that NO-producing neurons maintain and facilitate hyperalgesia [39,59]. NADPH-d active cells are found in the superficial dorsal horn (Laminae I–III) and pericentral region [38,51]. Although the majority of Fos immunoreactive nuclei do not colocalize with NOS/NADPH-d, a functional relationship between these molecules has been demonstrated [34,50]. NADPH-d histochemical activity is up-regulated in the Lamina I–III and central canal area following peripheral inflammation, axotomy and spinal cord lesion [8,51,59]. Although there is no information about GC effects on NADPH-d in the spinal cord, data collected in other systems agree that GC down-regulate this enzyme [35,49,56,62]. Thus, the possibility exists that NADPH-d and Fos may constitute important targets of GC action during acute SCI.

Therefore, the objectives of this investigation were: first, to determine changes in Fos and NADPH-d staining at several time points after SCI and, second, to asses the response of these parameters to acute (2–4 h) or sustained (up to 2 days) administration of the GC dexamethasone (DEX). To the best of our knowledge, modulation of Fos and NADPH-d by GC are still unexplored for the injured spinal cord. Results from this study have appeared in part in abstract form [24].

2. Materials and methods

2.1. Experimental animals

Male Sprague–Dawley rats (250–300 g) were deeply anesthetized with ketamine (60 mg/kg, i.p.). In sham-operated controls, soft tissues were cut and then sutured. For SCI, a careful laminectomy exposed the spinal cord, which was completely transected at the thoracic level (T7–T8) [21–23]. A group of rats with SCI received saline vehicle or 5 mg/Kg of DEX at 5 min post-lesion and sacrificed 2 or 4 h after surgery (N=4–6 animals per each time point). Another experimental group of SCI rats received vehicle or intensive DEX treatment 5 min, 6 h, 18 h and 46 h post-lesion and were sacrificed 48 h after surgery, i.e., 2 h after the last DEX injection (N=4–6 rats per each time point). Corresponding sham-operated controls for both experiments received either vehicle or DEX. DEX dosage was based on full occupancy of GR and maximal stimulation of spinal cord enzymes responding to GC treatment after SCI [19,45]. Regions analysed included the superficial dorsal horn (Laminae I–III) and the area around the central canal (Lamina X) at the L3–L5 level below the lesion site. These areas are known to display neuronal Fos immunoreactive nuclei and NADPH-d activity in response to SCI and sensory stimulation [31,59,60]. All protocols followed the Guide for the Use and Care of Laboratory Animals (National Institute of Health Guide, Instituto de Medicina y Biología Experimental, Assurance Certificate N A5072-01) and were approved by the Institute's Animal Care and Use Committee. Efforts were made to minimize animal suffering and to reduce the number of lesioned animals to a minimum [21–23].

2.2. Fos immunoreactivity

Animals anesthetized with ketamine (60 mg/kg, i.p.) were perfused intracardially with 0.9% NaCl followed by freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). The spinal cords were carefully dissected out and stored in the same fixative for 3 h (4°C). After post-fixation, spinal cord segments were cryoprotected in 20% sucrose with the same phosphate buffer and stored overnight at 4°C. The L3–L5 enlargements below the lesion site or a comparable region in sham-operated animals were embedded in Tissue-Tek (OCT compound, Miles Inc., USA). Transverse sections (15 μm) were cut on a cryostat maintained at −22°C. Slices were placed on gelatine-coated slides and fixed by immersion over 6 min in freshly prepared 2% paraformaldehyde at 4°C. For Fos immunostaining [67], tissue

sections were sequentially exposed to 10% goat serum for 10 min at room temperature, and then to the primary antibody raised against Fos protein (H-125 polyclonal rabbit antiserum, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:2000 dilution in 0.15% Triton X-100 at room temperature overnight. Sections were then washed twice with PBS and incubated in goat anti-rabbit serum (1:200) and 0.15% Triton X-100 for 1 h. This step was followed by three washes in PBS and processed using the Vectastain ABC kit (Vector Labs., Burlingame, CA, USA). Development was carried out using 1 mg/ml diaminobenzidine containing 0.01% H_2O_2. The chromogenic reaction was monitored microscopically. Omission of the primary antibody blunted the immunocytochemical signal.

2.3. NADPH-d histochemistry

Animal treatment was similar to that described for Fos immunodetection. A slight modification of the method of Vincent and Kimura [61] was used to determine NADPH-d activity [25,33]. The latter is based on reduction of nitroblue tetrazolium to its insoluble formazan reaction product with purple–black staining of neuronal somata and fibres. Cryostat sections (16 μm) obtained at the L3–L5 level below the lesion site were fixed by immersion in 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, over 6 min at 4°C. After fixation, the sections were rinsed twice in PBS and incubated in a solution containing 0.2 mg/ml of nitroblue tetrazolium (Sigma Chemicals, St. Louis, USA, N-6876), 2.7 mg/ml ℓ-malic acid (Sigma, M-9136), 1 mg/ml of β-NADPH (Sigma, N-1630) and 0.3% Triton X-100 dissolved in 0.1 M Tris–HCl buffer (pH 7.4). After keeping the reaction in the darkness over 60 min at 37°C, it was stopped by two washes in PBS at room temperature. Sections were then dehydrated briefly in ethanol, air dried and coverslipped with Permount. In order to test for possible non-specific reactions, some spinal cord sections were incubated in the same solution but without the substrate β-NADPH or the electron acceptor nitroblue tetrazolium. Omitting either β-NADPH or nitroblue tetrazolium in the incubation solution completely blocked neuronal somata and fiber staining.

2.4. Quantitative analysis

A computer-assisted image analysis system (Bioscan Optimas, Edmonton, WA, USA) [16,55] was used to determine the number of neurons exhibiting NADPH-d histochemical staining or Fos immunoreactive nuclei. Ten random sections from each animal were observed at 200 and 400× magnification with an Olympus optic microscope equipped with a VT-C33ON videocamera. For the dorsal horn, the area comprising Lamina I–II and outer Lamina III of the lumbar spinal cord [40] at the L3–L5 level was analysed. For Lamina X, neurons were counted within a 300-μm radius of the center of the central canal [41]. This area is rich in nociceptive and opioid-producing neurons [41,42]. Neuronal-like profiles showing NADPH-d activity or brown nuclear staining in the case of Fos immunoreactivity were counted under bright field illumination in at least ten randomn sections per animal. In addition, the relative density of the histochemical reaction product in the superficial dorsal horn was measured as described by Traub et al. [59]. For this purpose, digitized images of tissue sections from all experimental groups were displayed upon the video screen under identical lighting conditions and processed at the same time. Then the program calculated the average optical density of the pixels contained within the outlined area (comprising Laminae I–III) by using a 256-unit gray-level scale. As previously described, cell body labeling within the measured areas did not affect the main density reading of that area [59]. Results were expressed as the inverse logarithm of gray intensity per area (ILIGV/area: LIGV), a parameter proportional to the unweighted average optical density [55].

2.5. Statistical analysis

All data are presented as mean numerical density, i.e., number of NADPH-d histochemically stained neurons per mm^2 or Fos immunoreactive nuclei per mm^2. This method is more precise than comparing the number of neurons per section, which depends on the rostrocaudal level of sampling. For each rat, data obtained from at least ten random sections at the L3–L5 spinal cord level were pooled for the individual animal (N=4–6 animals per group per time point). Data were analyzed by analysis of variance (ANOVA) to reveal any differences between treatments and time conditions. When significant effects arose from the variance analyses, Newman–Keuls post hoc analyses were used to determine group differences for the variable. Statistical significance was established at $P<0.05$.

3. Results

3.1. Fos immunoreactivity

The effects of SCI and DEX treatment on Fos expression were determined in the superficial dorsal horn Laminae I–III and central canal Lamina X of the lumbar enlargement located below the lesioned site (Fig. 1A and B). In agreement with previous reports [30,52,54], constitutive Fos expression was weak to absent in sham-control animals (Figs. 1A and B and 2A and B). Fos expression in the superficial dorsal horn was altered by SCI at all time periods studied. Thus, the number of Fos positive nuclei/mm^2 was significantly increased over controls at 2 h ($P<0.01$), 4 h ($P<0.001$) and 48 h ($P<0.01$) after injury (Fig. 1A). This result agrees with

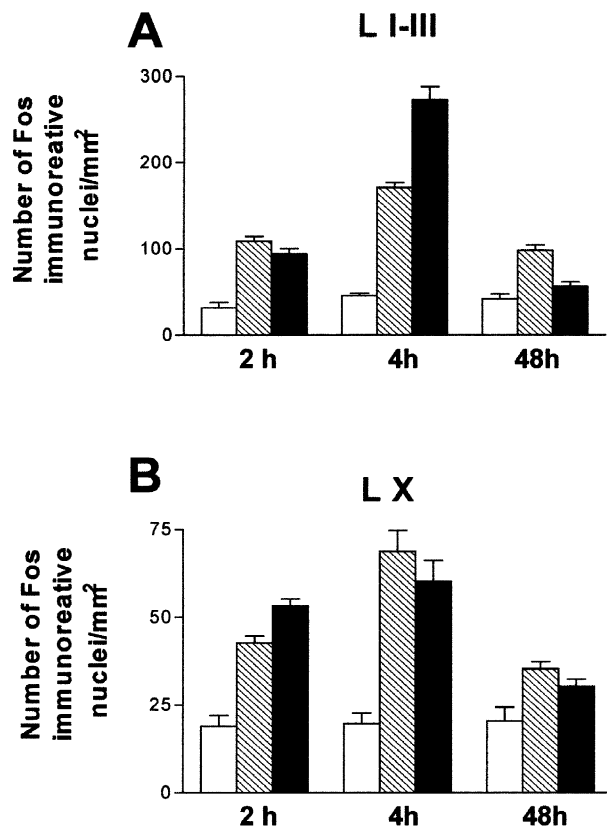

Fig. 1. Fos immunoreactivity after spinal cord injury (SCI) and dexamethasone treatment (DEX). Upper graph (A): number of Fos positive nuclei/mm^2 in Laminae I–III from sham-operated rats (open columns), rats with SCI (hatched columns) and rats with SCI receiving DEX (dark columns). Statistical analysis: SCI higher than sham-operated group at 2 h ($P<0.01$), 4 h ($P<0.001$) and 48 h ($P<0.01$). At the 4-h time-point, the SCI group receiving DEX were higher than the SCI group receiving vehicle ($P<0.001$). Lower graph (B): data from the pericentral area (Lamina X) from the groups depicted in the upper graph. Statistical analysis: SCI higher than sham-operated group at 2 h ($P<0.01$), 4 h ($P<0.01$) and 48 h ($P<0.05$). However, SCI receiving DEX similar to SCI group receiving vehicle at all time points.

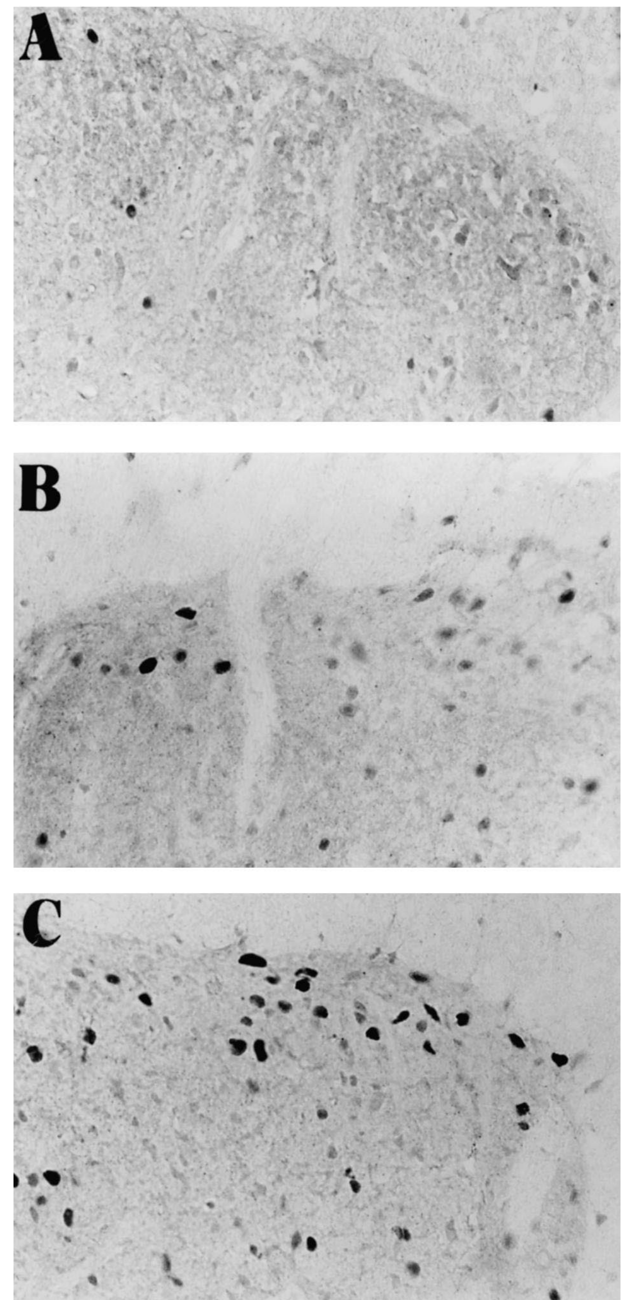

Fig. 2. Typical photomicrographs showing Fos immunoreactive cell nuclei in the superficial dorsal horn (Laminae I–III). In sham-operated rats only a few stained cells were observed (A), whereas many Fos immunopositive nuclei appeared 4 h after spinal cord injury (B). The dexamethasone treated rats showed higher Fos induction and staining intensity than sham-operated or untreated SCI rats (C). Magnification: 400×.

previous data in spinal cord injured rats [52]. As shown in Fig. 2B, strongly stained Fos positive nuclei were mainly observed 4 h after SCI (Fig. 2B). Glucocorticoid treatment of the lesioned group further enhanced the number of Fos immunoreactive nuclei in Lamina I–III by 4 h as compared to sham-operated controls ($P<0.001$) and to rats with SCI receiving vehicle ($P<0.001$) (Figs. 1A and 2C). However, Fos induction caused by DEX treatment at 2 and 4 h sharply declined after 2 days of injury, remaining at this time point in the range of sham-operated rats (Fig. 1A). To distinguish between the effects of the SCI alone and the DEX treatment, the effects of DEX alone was studied in uninjured control animals. However, no differences were found in the number of Fos positive nuclei/mm^2 of Lamina I–III between sham-operated controls (47.6±3.0), and controls receiving DEX and killed at early time periods (53.1±6.8) or at 48 h (52.1±4.3).

In a similar manner to the results of Laminae I–III, SCI significantly enhanced Fos expression over controls in the area around the central canal at 2 h ($P<0.01$), 4 h ($P<0.01$) and 48 h ($P<0.05$) (Fig. 1B). In the 2 day group, this effect appeared lower than in rats examined 2 and 4 h after SCI. DEX treatment was inactive at all time points. Thus, differences existed in steroid regulation of early gene expression between the dorsal horn and the central canal,

in that GC up-regulated Fos 4 h after SCI in Lamina I–III but not in Lamina X.

3.2. NADPH-d histochemistry

NADPH-d histochemistry revealed that SCI also produced changes in the number of stained neurons/mm^2 of the dorsal horn and central canal area located below the lesion site (Fig. 3A and B). In rats with SCI receiving vehicle, a significant increased number of dorsal horn neurons exhibiting NADPH-d staining by 2, 4 and 48 h after injury was obtained compared to sham-operated controls ($P<0.01$ in all cases; Fig. 3A). As opposed to its stimulatory effect on Fos expression, DEX administration significantly decreased the number of NADPH-d positive neurons at all times points examined, i.e., 2 h, 4 h and 2 days after SCI ($P<0.01$ or less at all time points). After DEX treatment, the number of NADPH-d positive neurons returned to near sham-control levels (Fig. 3A). As in the Fos study, we also carried out experiments to elucidate the effects of DEX alone in uninjured control animals. Whereas the number of NADPH-d active cells/mm^2 of Laminae I–III was 50±1.7 in the sham-group, the sham plus DEX group killed 48 h afterwards showed a comparable number of stained cells (55.9±5.5).

In addition to neurons, NADPH-d activity was also measured in cell processes (neuropil) extending as a countinuous band across the dorsal horn (Fig. 4). In this case, no changes were detected in neuropil histochemical staining density at 2 and 4 h after injury, but a delayed increase was obtained at 48 h ($P<0.01$ vs. sham-operated group) (Fig. 4). However, as in the case of positively stained neurons, steroid administration to rats with SCI returned the enhanced NADPH-d neuropil histochemical staining to sham-control levels (Fig. 4). The photomicrographs of Fig. 5 show the NADPH-d neuropil staining from a sham-operated rat (A), the increment after 2 days of SCI (B) and its reversal after GC treatment (C).

Fig. 3B also describes the effects of SCI on the number of NADPH-d positive neurons/mm^2 in Lamina X. No changes in this parameter were detected in the central canal area 2 or 4 h after injury, although a delayed increase was observed by 2 days after SCI (Fig. 3B). As in the case of the dorsal horn Laminae I–III, DEX treatment decreased the number of NADPH-d positive neurons to levels of sham-operated rats examined at this time point (Fig. 3B). Typical light-field photomicrographs of NADPH-d positive neurons denote few neurons around the central canal in a sham-control rat (Fig. 6A), an increased number of active neurons 2 days after SCI (Fig. 6B) and its reduction following glucocorticoid treatment (Fig. 6C).

Finally, a study was carried out to determine if Fos immunoreactivity colocalized with NADPH-d histochemis-

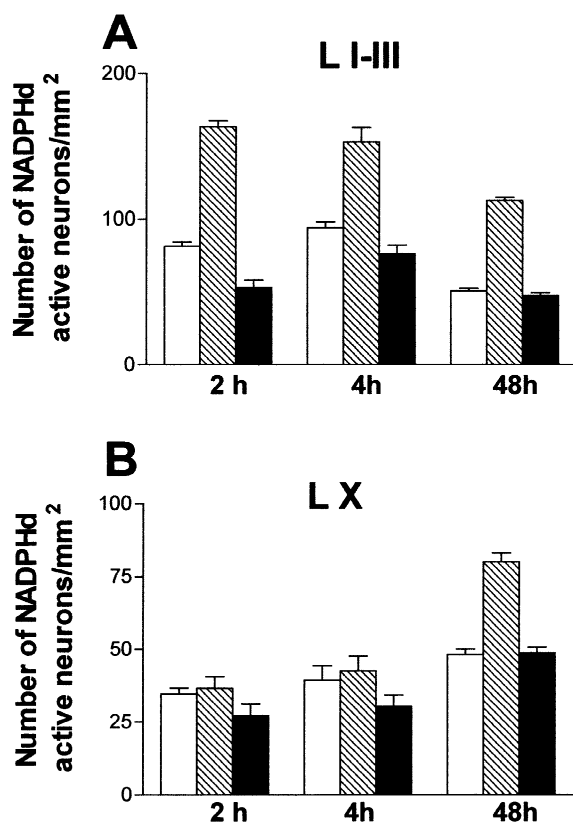

Fig. 3. NADPH-diaphorase active neurons/mm^2 after SCI and DEX treatment. Group labeling as in Fig. 1. Upper graph (A): NADPH-d in Laminae I-III. Statistical analysis: SCI (hatched columns) higher than sham-operated group (open columns) at all time points (2 h: $P<0.01$; 4 h: $P<0.01$ and 48 h: $P<0.01$). SCI group receiving DEX (dark columns) lower than SCI receiving vehicle at all time points (2 h: $P<0.01$; 4 h: $P<0.01$and 48 h: $P<0.01$). Lower graph (B): data from the pericentral area (Lamina X). Statistical analysis: at 48 h, SCI receiving vehicle was higher than sham-operated group ($P<0.05$) and SCI group receiving DEX ($P<0.05$).

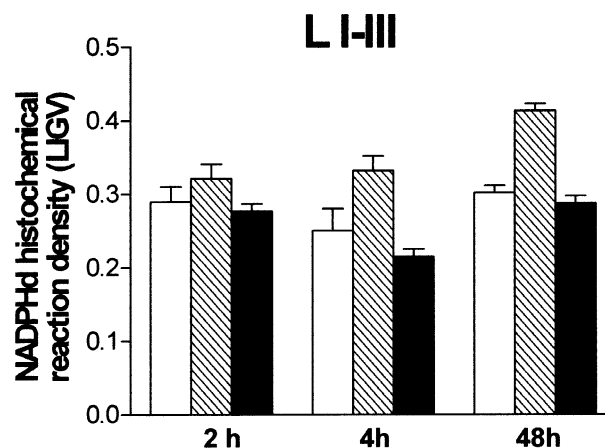

Fig. 4. NADPH-diaphorase activity in the superficial dorsal horn of sham-operated rats (open columns), rats with SCI (hatched columns) and in SCI rats receiving DEX (dark columns). The histochemical reaction is expressed as relative optical intensity per area (LIGV) [59]. Statistical analysis: at 48 h, SCI receiving vehicle was higher than sham-operated group ($P<0.01$) and SCI group receiving DEX ($P<0.01$).

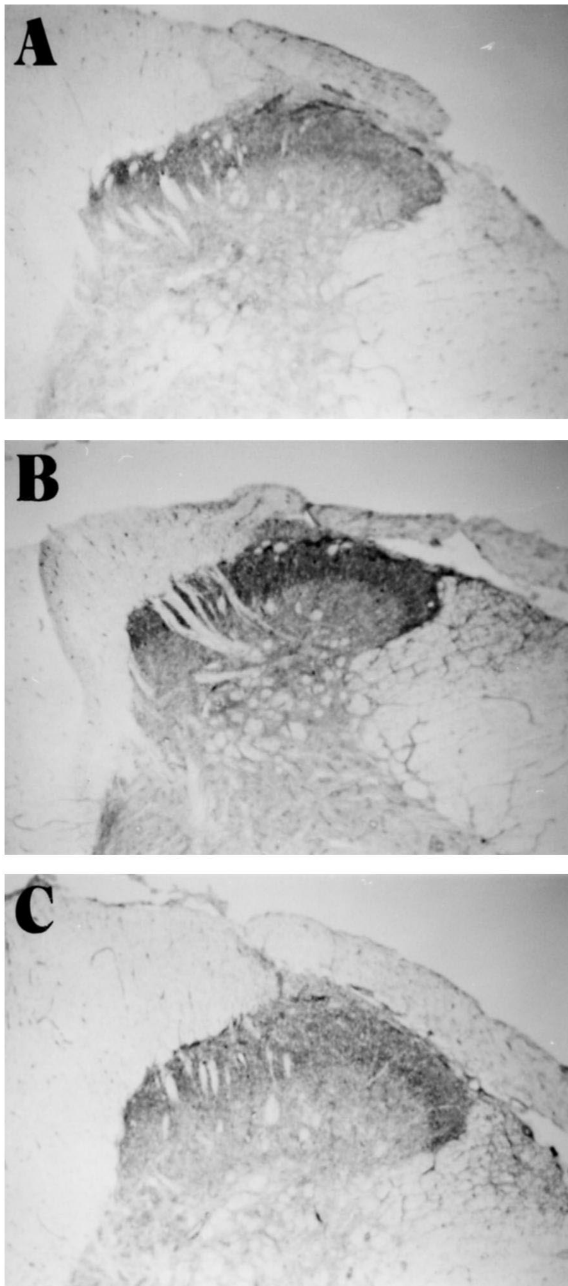

Fig. 5. Typical photomicrographs representing NADPH-diaphorase staining of neuropil plus neurons in the dorsal horn. Two days after sham-operation, a moderate basal staining intensity was obtained in sham-operated rats (A), which was substantially increased after spinal cord lesion (B). In spinal cord injured rats receiving intensive dexamethasone treatment over 2 days, staining intensity was attenuated (C) compared to injured rats receiving vehicle. Magnification: 100×.

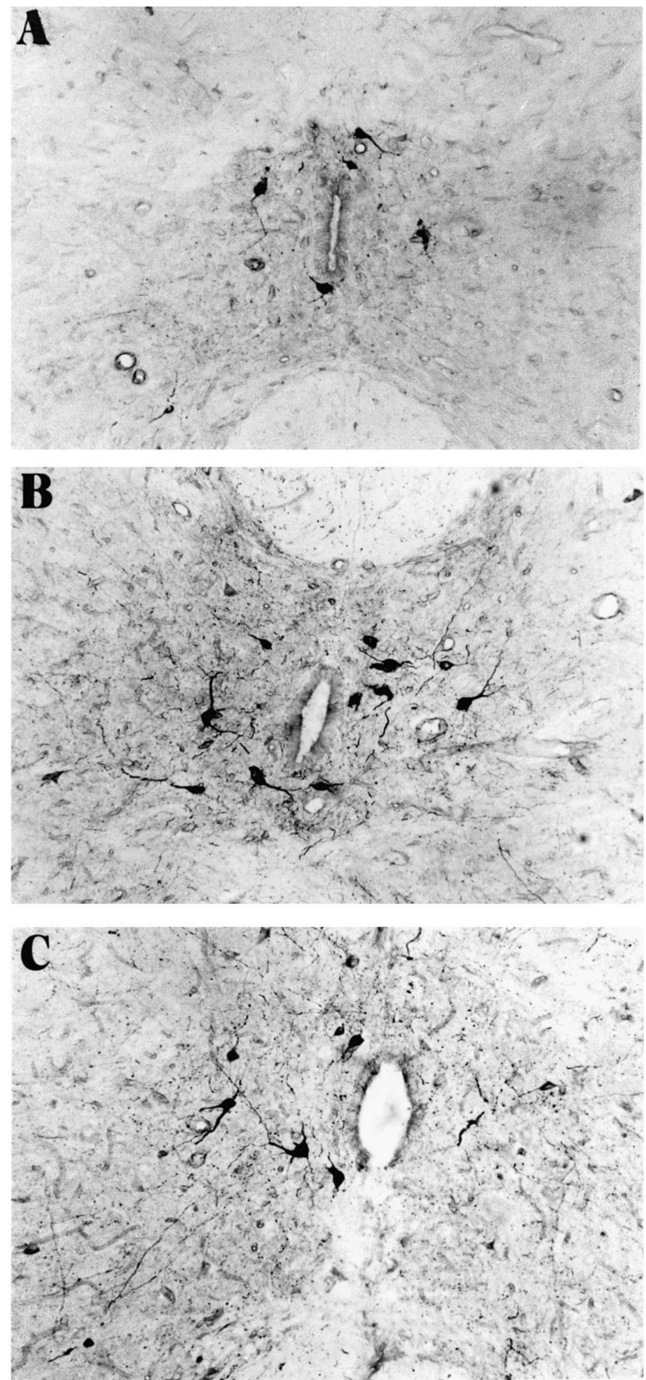

Fig. 6. NADPH-diaphorase histochemical staining in the central canal area (Lamina X). A low number of NADPH-diaphorase positive neurons characterized the pericentral area in sham-operated rats (A). After spinal cord injury, increased number of histochemically active neurons with long cell processes appeared (B). Instead, in spinal cord injured rats treated with dexamethasone (C) the photomicrograph resembled the sham-operated animal. Magnification: 200×.

try in neurons of the dorsal horn and central canal. However, no colocalization was found in Laminae I–III at any time point after SCI with or without DEX treatment. In Lamina X, colocalization at 2 h was only 12.3±2.3% of the total number of counted neurons without and 13.8±3.3% with DEX treatment. At 4 h the corresponding figures were 13.3±5.7 and 11.4±2.0%, respectively. At 48 h colocalization analysis was not possible due to the scarce number of Fos+nuclei. These data confirmed that Fos and NADPH-d were predominantly expressed in different cell populations [50].

4. Discussion

In this study we describe differential effects of DEX on Fos expression and NADPH-d histochemical staining in spinal cord injured rats. In the doses given, DEX maximally occupy GC receptors and induce GC-sensitive enzymes [19–21,45]. It should be noted that DEX and not methylprednisolone was used in this work, in spite of the fact that high doses of the later are currently in use for spinal cord injury [3,27]. However, use of DEX allowed a direct comparison of effects in sensory areas with those already reported in ventral horn motoneurons [21–23]. Also, the methylprednisolone mechanism of action may be related to its strong antioxidant effects [5,27,28], while DEX effects on Fos and NADPH-d require gene transcription [7,10,35,56]. In this case, the use of a potent GR agonist such as DEX seemed desirable.

In agreement with previous data [52], low to undetectable levels of Fos immunoreactive nuclei were found in spinal cord neurons within Laminae I–III and the pericentral area of sham-operated animals. However, a high number of Fos immunoreactive nuclei appeared in these Laminae by 2 h after SCI, reaching maximal levels of expression at 4 h after injury. This effect may be caused by interruption of supraspinal inhibitory pathways originating in the lower brain stem and upper spinal cord or from loss of a descending facilitatory effect on local inhibitory mechanisms [17,48,52]. However, it has also been considered that Fos predicts neuronal activation in Laminae I–III and Lamina X in response to sensory and noxious stimulation, because overexpression of this immediate early gene is found in experimental models of hyperalgesia and allodynia [17,30,31].

The molecular mechanism of Fos induction in the spinal cord may be coupled to early changes arising after trauma, such as stimulation of NMDA receptors [53,54,57,69]. In this regard, previous work from our laboratory demonstrated that glutamate binding to NMDA receptors was enhanced after SCI in Laminae I–III and Lamina X, i.e., areas showing up-regulation in Fos expression [21]. Interestingly, DEX treatment overstimulated NMDA receptor binding up to 6 h after transection, without further effects after 24 h [21]. A relationship between these factors emerges from the observation that Fos colocalizes in sensory neurons with NMDA receptors and also with GC receptors [10,54,57,69]. However, the use of the NMDA receptor antagonist ketamine for anesthesia, suggests that additional pathways for Fos stimulation not involving NMDA receptors may take place after SCI.

To account for the biological significance of Fos hyperexpression after GC therapy, it should be considered that in general Fos is a signal presumably involved in the cell body reaction to several factors, such as stress, axotomy or stimulation of neurotransmitter and peptide receptors [17,29,30,32]. Therefore, besides an hypothetical role in antinociception [17], neurons induced to express Fos could participate in other functions, including the regulation of sympathetic outflow [26,52]. In this way, restoration of blood pressure and sympathetic activity depressed by the state of spinal shock could be a function of GC therapy.

In our studies, SCI also produced a bilateral increase in the density of NADPH-d neuropil histochemical staining in the superficial layers of the dorsal horn and a significant increase in the number of NADPH-d positive neurons within Laminae I–III and Lamina X. These areas contain a moderate constitutive level of NADPH-d activity/NOS immunoreactivity [8,38,59]. Strong nociceptive input derived from spinal cord hemisection, root avulsion, hindpaw inflammation and axotomy produce robust increases in NADPH-d activity and/or NOS immunoreactivity as was the case with SCI [8,51,58,59,64]. However, these stimuli caused at the most a transient increase of a few hours in the enzyme, followed by a decrease persisting over many days [8]. The short latency of our reported increase in the number of NADPH-d positive neurons observed after SCI may be due to the activation of inactive enzyme in response to injury. However, the increased density of histochemical reaction in superficial neuropil 48 h after injury may be the result of the novo synthesis of the enzyme and its transport from the dorsal root ganglia to the spinal cord, although up-regulation of the enzyme activity in local interneurons is also possible [51]. A different time-dependent pattern was obtained in neurons within Lamina X, because a delayed increase of histochemically stained neurons was found 48 h after SCI. Although neurons around Lamina X present some similarities with peripheral dorsal horn neurons, differences also exist regarding their relationships to ascending/descending pathways and response to NO donors [41,47].

Our data also demonstrated that DEX exerted an inhibitory effect on the number of NADPH-d stained neurons in Lamina I–III and central canal area, elevated as a consequence of SCI. In this context, formation of nitric oxide due to this enzyme has been implicated in nociceptive transmission, because inhibition of its biosynthesis resulted in diminished response to pain [17]. Both natural and synthetic GC exert a negative regulation of this enzyme in brain and spinal cord motoneurons [25,49,56,62]. Thus, GC effects on NADPH-d activity after SCI may be somehow related to anti-hyperalgesic, anti-inflammatory effects of these hormones [1,14,43,68]. If sustained production of NO after SCI may be required for maintenance of experimentally-produced hyperalgesia [39], and if a lesion-induced increase in NO is likely to cause spontaneous pain [8], a blockade of NADPH-d by GC treatment would likely reduce pain excitatory neurotransmission at the spinal cord level. In molecular terms, GC action upon NADPH-d may bear a relation with steroid antiinflammatory and immunosuppressive effects, mainly achieved by transcriptional interference between GC receptors and NF-κB [63]. It is known, for example, that traumatic spinal

cord injury and DEX have opposite effects on NF-κB activation [2,63,68]. The fact that NOS gene expression follows in part NF-κB activation, and that both molecules colocalize in neurons [2], suggests that GC effects on NADPH-d/NOS may be secondary or closely related to GC repression of NF-κB activity. Thus, GC inhibition of NADPH-d activity, as shown in this work, and repression of NF-κB after traumatic spinal cord injury [68] may have important implications for the prevention of secondary neuronal death consequent to spinal cord trauma. Recently, DEX was also shown to reduce apoptosis in contused spinal cord [4]. Therefore, it is possible that inhibition of NFκB and NADPH-d are consequences of GC blockade of an apoptotic process.

Lastly, the differential effects of DEX on Fos and NADPH-d may rely on the fact that these molecules are present in different cell populations, as reported by others and also in the present work [34,50]. In this way, DEX acting on different sets of sensory neurons could become a positive (Fos) or negative (NADPH-d) modulator of Laminae I–III and pericentral neurons. Therefore, while DEX effects in ventral horn neurons could lead to a recovery of motor function, inhibition of pain-related mechanisms and restoration of sympathetic activity in sensory areas may constitute additional mechanisms contributing to recovery from the state of spinal shock and enhanced nociception which are undesirable effects of spinal cord injury [1,9,11,27,28,46].

Acknowledgements

This work was supported by grants from CONICET (PIP 4103 and PEI 03228/98), University of Buenos Aires (MT13), FONCYT (BID 802 OC AR PICT 97 05-00438), Fundación Barceló (Buenos Aires) and Beca Ramón Carrillo-Arturo Oñativia from the Minister of Health of Argentina.

References

[1] D. Bartholdi, M.E. Schwab, Methylprednisolone inhibits early inflammatory processes but not ischemic cell death after experimental spinal cord lesion in the rat, Brain Res. 672 (1995) 177–186.

[2] J.R. Bethea, M. Castro, R.W. Keane, T.T. Lee, W.D. Dietrich, R.P. Yezierski, Traumatic spinal cord injury induces nuclear factor-kB activation, J. Neurosci. 18 (1998) 3251–3260.

[3] M.B. Bracken, M.J. Shepard, W.F. Collins, T.R. Holford, D.S. Baskin, H.M. Eisenberg, E. Flamm, L. Leo-Summers, J. Maroon, L.F. Marshall, P.L. Perot, J. Piepmeir, V.K.H. Sonntag, F.C. Wagner, J.E. Wilberger, H.R. Winn, A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal cord injury, New England J. Med. 322 (1990) 1405–1411.

[4] C. Brandoli, B. Shi, B. Pflug, P. Andrews, J.R. Wrathall, I.I. Mochetti, Dexamethasone reduces the expression of p75 neurotrophin receptor and apoptosis in contused spinal cord, Mol. Brain Res. 19 (2001) 61–70.

[5] J.M. Braughler, E.D. Hall, Correlation of methylprednisolone levels in cat spinal cord with its effects on (Na, K-)ATPase, lipid peroxidation and alpha motor neuron function, J. Neurosurg. 56 (1982) 838–844.

[6] K.P. Briski, B.M. DiPasquale, E. Gillen, Induction of immediate-early genes in preoptic and hypothalamic neurons by the glucocorticoid receptor agonist, dexamethasone, Brain Res. 768 (1997) 185–196.

[7] J. Buritova, P. Honore, V. Chapman, J.M. Besson, Enhanced effect of co-administered dexamethasone and diclofenac on inflammatory pain processing and associated spinal c-fos expression in the rat, Pain 64 (1996) 559–568.

[8] P. Callsen-Cencic, U. Hoheisel, A. Kaske, S. Mense, S. Tenschert, The controversy about spinal neuronal nitric oxide synthase: under which conditions is it up- or downregulated? Tissue Res. 295 (1999) 183–194.

[9] A. Chen, X.M. Xu, N. Kleitman, M.B. Bunge, Methylprednisolone administration improves axonal regeneration into Schwann cell grafts in transacted adult rat thoracic spinal cord, Exp. Neurol. 138 (1996) 261–276.

[10] A. Cintra, C. Molander, K. Fuxe, Colocalization of Fos and glucocorticoid receptor-immunoreactivities is present only in a very restricted population of dorsal horn neurons of the rat spinal cord after nociceptive stimulation, Brain Res. 632 (1993) 334–338.

[11] A.F. De Nicola, Steroid hormones and neuronal regeneration, Adv. Neurol. 59 (1993) 199–206.

[12] R. Dubner, M.A. Ruda, Activity-dependent neuronal plasticity following tissue injury and inflammation, Trends Neurosci. 15 (1992) 96–103.

[13] G.E. Duncan, W.E. Stumpf, Target neurons for (3H)-corticosterone in the rat spinal cord, Brain Res. 307 (1984) 321–326.

[14] S.H. Ferreira, F.Q. Cunha, B.B. Lorenzetti, M.A. Michelin, M. Perretti, R.J. Flower, S. Poole, Role of lipocortin-1 in the anti-hyperalgesic actions of dexamethasone, Br. J. Pharmacol. 121 (1997) 883–888.

[15] M. Ferrini, S. Gonzalez, T. Antakly, A.F. De Nicola, Immunocytochemical localization of glucocorticoid receptors in the spinal cord: effects of adrenalectomy, glucocorticoid treatment, and spinal cord transection, Cell. Mol. Neurobiol. 13 (1993) 387–397.

[16] M. Ferrini, G. Piroli, M. Frontera, A. Falbo, A. Lima, A.F. De Nicola, Estrogens normalize the hypothalamic-pituitary-adrenal axis response to stress and increase glucocorticoid receptor immunoreactivity in hippocampus of aging male rats, Neuroendocrinology 69 (1999) 129–137.

[17] S. Fürst, Transmitters involved in antinociception in the spinal cord, Brain Res. Bull. 48 (1999) 129–141.

[18] K. Fuxe, A. Harfstrand, L.F. Agnati, Z.-Y. Yu, A. Cintra, A.C. Wikstrom, S. Okret, E. Cantoni, J.-A. Gustafsson, Immunocytochemical studies on the localization of glucocorticoid receptor immunoreactive cells in the lower brain stem and spinal cord of the male rat using a monoclonal antibody against rat liver glucocorticoid receptor, Neurosci. Lett. 60 (1985) 1–6.

[19] S. Gonzalez, D.F. Moses, A.F. De Nicola, Glucocorticoid receptors and enzyme induction in the spinal cord of rats: effects of acute transection, J. Neurochem. 54 (1990) 834–840.

[20] S. Gonzalez, M. Ferrini, H. Coirini, M.C. Gonzalez Deniselle, A.F. De Nicola, Regulation of flunitrazepam binding in the dorsal horn of the spinal cord by adrenalectomy and corticosteroids, Brain Res. 589 (1992) 97–101.

[21] S.L. González, H. Coirini, M.C. González Deniselle, S. González, R. Calandra, A.F. De Nicola, Time-dependent effects of dexamethasone on glutamate binding, ornithine decarboxylase activity and polyamine levels in the transected spinal cord, J. Steroid Biochem. Mol. Biol. 55 (1995) 85–92.

[22] S.L. González, C. Grillo, M.C. González Deniselle, A. Lima, B.S. McEwen, A.F. De Nicola, Dexamethasone up-regulates mRNA for Na, K-ATPase in some spinal cord neurons after cord transection, Neuroreport 7 (1996) 1041–1044.

[23] S.L. González, F. Saravia, M.C. González Deniselle, A. Lima, A.F. De Nicola, Glucocorticoid regulation of motoneuronal parameters in rats with spinal cord injury, Cell. Mol. Neurobiol. 19 (1999) 597–611.
[24] S.L. González, M.C. González Deniselle, A. Lima, R. Guennoun, A.F. De Nicola, Glucocorticoid regulation of Fos-like immunoreactivity in the normal and injured spinal cord, in: Society for Neuroscience 30th Annual Meeting, New Orleans, LA, USA, 2000, p. 510.
[25] M.C. González Deniselle, S.L. González, A. Lima, G. Wilkin, A.F. De Nicola, The 21-aminosteroid U-74389F attenuates hyperexpression of GAP-43 and NADPH-diaphorase in the spinal cord of Wobbler mouse, a model for amyotrophic lateral sclerosis, Neurochem. Res. 24 (1999) 1–8.
[26] O.M. Gootman, M.I. Cohen, Sympathetic rhythms in spinal cats, J. Auton. Nerv. Syst. 3 (1981) 379–387.
[27] E.D. Hall, The neuroprotective pharmacology of methylprednisolone, J. Neurosurg. 76 (1992) 13–22.
[28] E.D. Hall, Neuroprotective actions of glucocorticoid and nonglucocorticoid steroids in acute neuronal injury, Cell. Mol. Neurobiol. 13 (1993) 415–432.
[29] J.A. Harris, Using c-fos as a neuronal marker of pain, Brain Res. Bull. 45 (1998) 1–8.
[30] P. Hughes, M. Dragunow, Induction of immediate early genes and the control of neurotransmitter-regulated gene expression within the nervous system, Pharmacol. Rev. 47 (1995) 133–178.
[31] S.P. Hunt, A. Pini, G. Evan, Induction of c-fos-like protein in spinal cord neurons following sensory stimulation, Nature 328 (1987) 632–634.
[32] K. Kovács, c-Fos as a transcription factor: a stressful (re)view from a functional map, Neurochem. Int. 33 (1998) 287–297.
[33] F. Labombarda, S.L. Gonzalez, P. Roig, A. Lima, R. Guennoun, M. Schumacher, A.F. De Nicola, Modulation of NADPH-diaphorase and glial fibrillary acidic protein by progesterone in astrocytes from normal and injured rat spinal cord, J. Steroid Biochem. Mol. Biol. 73 (2000) 159–169.
[34] J.H. Lee, R.H. Price, F.G. Williams, B. Mayer, A.J. Beitz, Nitric oxide synthase is found in some spinothalamic neurons and in neuronal processes that appose spinal neurons that express Fos induced by noxious stimulation, Brain Res. 608 (1993) 324–333.
[35] M.O. Lopez Figueroa, K. Itoi, S.J. Watson, Regulation of nitric oxide synthase messenger RNA expression in the rat hippocampus by glucocorticoids, Neuroscience 87 (1998) 439–446.
[36] L.G. Luo, I.M.D. Jackson, Glucocorticoids stimulate TRH and c-fos/c-jun gene expression in cultured hypothalamic neurons, Brain Res. 791 (1998) 56–62.
[37] L.N.J.L. Marlier, T. Csikos, N. Rebaudengo, P. Borboni, F.R. Patacchioli, L. Angelucci, A. Privat, R. Lauro, Distribution of glucocorticoid receptor mRNA in the rat spinal cord, Neuroreport 6 (1995) 2245–2249.
[38] J. Marsala, M. Marsala, I. Vanicky, Y. Taira, Localization of NADPHd-exhibing neurons in the spinal cord of the rabbit, J. Comp. Neurol. 406 (1999) 263–284.
[39] S.T. Meller, G.F. Gebhart, Nitric oxide (NO) and nociceptive processing in the spinal cord, Pain 52 (1993) 127–136.
[40] C. Molander, Q. Xu, G. Grant, The cytoarchitectonic organization of the spinal cord in the rat. I. The lower thoracic and lumbosacral cord, J. Comp. Neurol. 230 (1984) 133–141.
[41] R.L. Nahin, A.M. Madsen, G.J. Giesler, Anatomical and physiological studies of the gray matter surrounding the spinal cord central canal, J. Comp. Neurol. 220 (1983) 321–335.
[42] A.P. Nicholas, X. Zhang, T. Hökfelt, An immunocytochemical investigation of the opioid cell column in lamina X of the male rat lumbosacral spinal cord, Neurosci. Lett. 270 (1999) 9–12.
[43] D. Nohr, M.K. Schafer, S. Persson, H. Romeo, F. Nyberg, C. Post, G. Ekstrom, G.E. Weihe, Calcitonin gene-related peptide gene expression in collagen induced arthritis is differentially regulated in primary afferents and motoneurons: influence of glucocorticoids, Neuroscience 93 (1999) 759–773.
[44] J. Orendácová, M. Marsala, I. Sulla, J. Kafka, P. Jalc, D. Cizková, Y. Taira, J. Marsala, Incipient cauda equina syndrome as a model of somatovisceral pain dogs: spinal cord structures involved as revealed by the expression of c-fos and NADPH-diaphorase activity, Neuroscience 95 (2000) 543–557.
[45] E. Orti, D.F. Moses, C. Grillo, A.F. De Nicola, Glucocorticoid regulation of glycerolphosphate dehydrogenase and ornithine decarboxylase activities in the spinal cord of the rat, J. Neurochem. 48 (1987) 425–431.
[46] M. Oudega, C.G. Vargas, A.B. Weber, N. Kleitman, M.B. Bunge, Long-term effects of methylprednisolone following transection of adult rat spinal cord, Eur. J. Neurosci. 11 (1999) 2453–2464.
[47] U. Pehl, H.A. Schmidt, Electrophysiological responses of neurons in the rat spinal cord to nitric oxide, Neuroscience 77 (1997) 563–573.
[48] L.R. Poree, L.P. Schramm, Role of cervical neurons in propriospinal inhibition of thoracic dorsal horn neurons, Brain Res. 599 (1992) 302–308.
[49] M.W. Radomski, R.M.J. Palmer, S. Moncada, Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells, Proc. Natl. Acad. Sci. 87 (1990) 10043–10047.
[50] L. Rodella, R. Rezzani, C. Agostini, R. Bianchi, Expression of NADPH-diaphorase and colocalization with Fos in the brain neurons of the rat following visceral noxius stimulation, Brain Res. 834 (1999) 173–177.
[51] M.A. Ruda, D. Besse, S. Inagaki, M. Deleón, K. Ren, Nitric oxide expression and regulation in the dorsal root ganglion and spinal cord, Ann. NY Acad. Sci. 738 (1994) 181–190.
[52] D.A. Ruggiero, M. Anwar, J. Kim, A.L. Sica, N. Gootman, P.M. Gootman, Induction of c-fos gene expression by spinal cord transection in the rat, Brain Res. 763 (1997) 21–29.
[53] T.E. Salt, P.L. Herring, Excitatory amino acid transmitter function in mammalian central pathways, in: H.V. Wheal, A.M. Thomson (Eds.), Excitatory Amino Acids and Synaptic Transmission, Academic Press, New York, 1991, pp. 155–167.
[54] J. Sandkuhler, A.C. Treier, X.G. Liu, M. Ohnimus, The massive expression of c-Fos protein in spinal cord dorsal horn neurons is not followed by long-term changes in spinal nociception, Neuroscience 73 (1996) 657–666.
[55] F. Saravia, C. Grillo, M. Ferrini, P. Roig, A. Lima, E.R. de Kloet, A.F. De Nicola, Changes of hypothalamic and plasma vasopressin in rats with deoxycorticosterone-acetate induced salt appetite, J. Steroid Biochem. Mol. Biol. 70 (1999) 47–57.
[56] P.M. Schwarz, B. Gierten, J.-P. Boissel, U. Forstermann, Expressional down-regulation of neuronal type nitric oxide synthase I by glucocorticoids in N1E-115 neuroblastoma cells, Mol. Pharmacol. 54 (1998) 258–263.
[57] Y.-X. Thao, Z.Q. Zhao, NMDA receptors mediating Fos expression in rat spinal cord induced by subcutaneous injection of formalin, Acta Pharmacol. Sinica 19 (1998) 506–509.
[58] R.J. Traub, A. Solodkin, G.F. Gebhart, NADPH-diaphorase histochemistry provides evidence for a bilateral, somatotopically inappropriate response to unilateral hindpaw inflammation in the rat, Brain Res. 647 (1994) 113–123.
[59] R.J. Traub, A. Solodkin, S.T. Meller, G.F. Gebhart, Spinal cord NADPH-diaphorase histochemical staining but not nitric oxide synthase immunoreactivity increases following carrageenan-produced hindpaw inflammation in the rat, Brain Res. 668 (1994) 204–210.
[60] L. Urban, S.W.N. Thompson, A. Dray, Modulation of spinal excitability: co-operation between neurokinins and excitatory amino acids neurotransmitters, TINS 17 (1994) 432–438.
[61] S.R. Vincent, H. Kimura, Histochemical mapping of nitric oxide synthase in the rat brain, Neuroscience 46 (1992) 755–784.
[62] C.M. Weber, B.C. Eke, M.D. Maines, Corticosterone regulates heme

oxygenase-2 and NO synthase transcription and protein expression in rat brain, J. Neurochem. 63 (1994) 953–962.

[63] S. Wissink, E.C. van Heerde, B. van den Burg, P.T. van der Saag, A dual mechanism mediates repression of NF-kB activity by glucocorticoids, Mol. Endocrinol. 12 (1998) 355–363.

[64] W. Wu, Y. Li, F.P. Schinco, Expression of c-jun and neuronal nitric oxide synthase in rat spinal motoneurons following axonal injury, Neurosci. Lett. 179 (1994) 157–181.

[65] P. Yan, J. Xu, Q. Li, S. Chen, G.M. Kim, C.Y. Hsu, X.M. Xu, Glucocorticoid receptor expression in the spinal cord after traumatic injury in adult rats, J. Neurosci. 19 (1999) 9355–9363.

[66] L.-W. Yick, W. Wu, K.-F. So, S.Y. Wong, Time course of NOS expression and neuronal death in Clarke's nucleus following traumatic injury in adult rat spinal cord, Neurosci. Lett. 241 (1998) 155–158.

[67] Z. Xu, A.K. Johnson, Central renin injections: effects on drinking and expression of immediate early genes, Brain Res. 782 (1998) 24–35.

[68] J. Xu, G. Fan, S. Chen, Y. Wu, X.M. Xu, C.Y. Hsy, Methylprednisolone inhibition of TNF-alpha expression and NF-κB activation after spinal cord injury in rats, Mol. Brain Res. 59 (1998) 135–142.

[69] R.X. Zhang, H. Wang, M. Ruda, M.J. Ladarola, J.T. Qiao, c-Fos expression in NMDA receptor-contained neurons in spinal cord in a rat model of inflammation: a double immunocytochemical study, Brain Res. 795 (1998) 282–286.