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ORIGINAL PAPER

Progesterone Effects on Neuronal Ultrastructure and Expression of Microtubule-associated Protein 2 (MAP2) in Rats with Acute Spinal Cord Injury

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Abstract (1) Following acute spinal cord injury, progesterone modulates several molecules essential for motoneuron function, although the morphological substrates for these effects are unknown. (2) The present study analyzed morphological changes in motoneurons distal to the lesion site from rats with or without progesterone treatment. We employed electron microscopy to study changes in nucleus and cytoplasm and immunohistochemistry for the microtubule-associated protein 2 (MAP2) for changes in cytoskeleton. (3) After spinal cord injury, the nucleoplasm appeared more finely dispersed resulting in reduced electron opacity and the nucleus adopted an eccentric position. Changes of perikarya included dissolution of Nissl bodies and dissociation of polyribosomes (chromatolysis). After progesterone treatment for 3 days, the deafferented motoneurons now presented a clumped nucleoplasm, a better-preserved rough endoplasmic reticulum and absence of chromatolysis. Progesterone partially prevented development of nuclear eccentricity. Whereas 50% of injured motoneurons showed nuclear eccentricity, only 16% presented this phenotype after receiving progesterone. Additionally, injured rats showed reduced immunostaining for MAP2 in dendrites, pointing to cytoskeleton abnormalities, whereas progesterone treatment attenuated the injury-induced loss of MAP2. (4) Our data indicated that progesterone maintained in part neuronal ultrastructure, attenuated chromatolysis, and preclude the loss of MAP2, suggesting a protective effect during the early phases of spinal cord injury.

Keywords Chromatolysis · Electron microscopy · MAP2 · Motoneuron · Neuroprotection · Progesterone · Spinal cord injury

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Introduction

Progesterone exerts pleiotropic effects in the nervous system that extend far beyond its classical reproductive functions. Thus, in models of CNS injury and neurodegeneration, progesterone increases myelination, sustains neuronal viability, and reduces the inflammatory response (Azcoitia et al. 2003; De Nicola et al. 2006; Desarnaud et al. 1998; Ghoumari et al. 2003; Gonzalez et al. 2005; Gonzalez Deniselle et al. 2003; Grossman et al. 2004; Ibanez et al. 2003; Koenig et al. 1995; Magnaghi et al. 2001; Melcangi and Mensah-Nyagan 2006). These progesterone effects correlate with a better clinical outcome following brain trauma in rats and humans (Roof et al. 1997; Stein 2005; Wright et al. 2007) and spinal cord injury in rats (Thomas et al. 1999), although there is no consensus on this matter (Fee et al. 2007).

Previous work has shown that progesterone regulates some key features of neuronal function after a complete midthoracic transection of the rat spinal cord. In deafferented motoneurons, progesterone increases choline acetyltransferase immunoreactivity, mRNA expression levels for the Na,K-ATPase, and growth-associated protein, up-regulates the mRNA and protein expression of neuronal brain-derived neurotrophic factor and prevents the lesion-induced disruption of Nissl bodies (Gonzalez et al. 2004, 2005; Labombarda et al. 2002). Since the spinal cord expresses classical as well as membrane progesterone receptors (PR) (Labombarda et al. 2003), different mechanisms and signaling cascades may be involved in these hormonal effects.

Neuropathology of spinal cord injury develops in a time-dependent fashion. In the acute phase, extending over the first few days after trauma, neuronal damage results from ischemia, ion imbalances, cytotoxic edema, and increased oxidative stress (Bareyre and Schwab 2003). Secondary effects that may last for weeks include inflammation, excitototoxic damage, and neuronal death (Bareyre and Schwab 2003). The neuronal response to spinal injury, neurodegeneration, or axonal injury includes development of nuclear eccentricity and centrifugal disappearance of Nissl bodies with disruption of polyribosomes (chromatolysis) (Gonzalez et al. 2004; Nacimiento et al. 1995; Price and Porter 1972). Recent evidences point to nuclear eccentricity as a landmark of cell damage (Jamielson et al. 2007; Zeman et al. 2004). In addition, there are evidences for activation of apoptotic cascades after SCI (Bjugn et al. 1997; Hayashi et al. 1998; Springer et al. 1997) and of transsynaptic neurodegeneration in up to 25% of neurons distal to the lesion site (Eidelberg et al. 1989). These abnormalities may be due to a combination of factors, such as loss of neurotrophic support to the injured neurons, excitotoxic damage, and increased oxidative stress (Azbill et al. 1997; Gold et al. 1991; Gonzalez et al. 2005; Cragg 1970; Roof et al. 1997; Springer et al. 1997).

Either CNS trauma or neurodegeneration leads to cytoskeleton alterations affecting microtubules and neurofilaments, in particular the loss of microtubule-associated protein 2 (MAP2), a major component of the cytoskeleton (Kikuchi et al. 1999; McIlwain and Hoke 2005; Yu et al. 2000; Zhang et al. 2000). Interestingly, several reports suggest that MAP2 is a target of progesterone and other steroids. For example, progesterone stimulates neuronal MAP2 expression in cerebellar slices (Ghoumari et al. 2003) and this protein has been involved in the structural changes induced by estradiol and progesterone in the hippocampus (Reyna-Neyra et al. 2002). In addition, neurosteroids bind MAP2, and this interaction may induce the formation and stabilization of microtubules (Fontaine-Lenoir et al. 2006).

The present report studied the effects of progesterone treatment on structural alterations developing in motoneurons during the early phase (i.e., 3 days) of spinal cord injury. As already discussed, during this time window progesterone treatment maintains the expression of several molecules essential for motoneuron function and prevents demyelination (Gonzalez et al. 2004; Labombarda et al. 2002, 2006a). We thus compared the changes in the nucleus, the nucleolus and perikaryon emerging in spinal motoneurons after a complete spinal cord transection with or without a 3-day course of progesterone treatment. In addition, we quantified the nuclear eccentricity and MAP2 immunostaining density, as reliable markers of changes taking place at the neuronal and cytoskeleton/dendritic level, respectively. Our results showed that progesterone maintained in part neuronal ultrastructure, attenuated chromatolysis, and preclude the loss of MAP2.

Methods

Animals and Surgical Procedures

We used male Sprague–Dawley rats (250–300 g) deeply anesthetized with a mixture of ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Spinal cord injury was performed under an operating microscope by transecting the spinal cord at thoracic level (T10) with iridectomy scissors (Gonzalez et al. 2004; Labombarda et al. 2006a). We verified the completeness of the transection by passing the sharp edge of a 25-G needle through the lesion site. Gelfoam (Pharmacia & Upjohn, Kalamazoo, MI, USA) application was used to control bleeding and body temperature was maintained at 37°C. In sham-control animals, the spinal cord was not lesioned. A group of injured rats received vehicle or four injections of progesterone (Proluton, Schering Laboratories, Argentina; 4 mg/kg b.w.) at 1 h and again at 24, 48, and 72 h (s.c.) post-lesion. This protocol of steroid administration prevents neuronal loss after brain injury in rats, modulates neuronal parameters after spinal transection and improves clinical and histological outcome after spinal contusion (Labombarda et al. 2002; Roof et al. 1997; Thomas et al. 1999). Animals were killed 3 days after complete transection or sham operation.

Studies were carried out at lumbar L1 level caudal to the lesion site. Transection at T10 interrupts supraspinal descending tracts as well as propiospinal pathways to lumbar segments, leading to transsynaptic degeneration of motoneurons located below the lesion site (Eidelberg et al. 1989; Nacimiento et al. 1995). Animal procedures followed the Guidelines for the Care and Use of Laboratory Animals (National Institute of Health granted the Assurance Certificate N A5072-01 to Instituto de Biología y Medicina Experimental) and received approval of the Institute's Animal Care and Use Committee. Efforts were made to reduce animal discomfort and to keep the number of lesioned animals to a minimum.

Electron Microscopy

Animals were anesthetized and perfused transcardially with a solution containing 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Spinal cords were removed and small blocks of tissue obtained by cutting transverse sections of 2–3 mm maximum length, located at the L1 segment caudal to the lesion site or a comparable region of sham-operated animals. Blocks were immersed for 2 h in the same fixative and washed overnight in 0.1 M phosphate buffer. Tissue blocks containing the ventral horns were post-fixed in 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4, for 2 h and stained with 5% uranyl acetate. Afterwards, tissue blocks were dehydrated and flat embedded in Durcupan (Fluka Cemic AG, Sweden). Semithin sections (0.5–1 μm) were stained with toluidine blue for light microscopy observations. For electron microscopy, we obtained ultrathin sections (60–70 nm) with an Ultracut-E Reichert-Jung ultramicrotome (Vienna, Austria) from ventral horns containing large motoneurons, according to prior visualization in semithin sections. We examined sections stained with lead citrate at 4,000×, 8,000×, and 20,000× magnifications and photographed using a Zeiss 10C electron microscope. Digitalization of photographs and image contrast and brightness were adjusted electronically, using Adobe Photoshop 6.0 software.

Nissl Staining and Measurement of Nuclear Eccentricity

Anesthetized animals were first perfused with 0.9% (w/v) cold NaCl and then with Bouin's fixative made of saturated picric acid–formaldehyde–glacial acetic acid (3:2:0.2, v/v). Spinal segments were fixed in 10% (v/v) formalin for 48 h, dehydrated, and processed for standard paraffin embedding following a routine protocol (Chemicon Int., Temecula, CA, USA). Five-micrometer spinal sagital sections corresponding to L1 level were stained with 0.5% Cresyl Violet (Sigma), dehydrated in graded ethanols and xylene, and coverslipped with Permount. The study was restricted to the dorsolateral group of ventral horn neurons of Lamina IX (that apply to limb muscles) in order to obtain as homogeneous a cell population as possible.

The methods used to derive quantitative data were as follows: an image analysis program (Optimas, Bioscan 6.5 version) traced the major and minor axis of 200 ventral horn neuronal outlines on the control and experimental groups. We took the mid-point between the ends of the major axis as an approximation of the center of the cell body. The latter point is necessary for the measurement of nuclear eccentricity.

This program also drew the cell radius passing through the center of the nucleus. Nuclear eccentricity was calculated by the method of Barr and Hamilton (1948) using the formula $[AC/(AB - CD)] \times 100\%$, where A, B, C, and D are points along a line from the center of the cell body (A) through the center of the nucleus (C) to the cell periphery (B). AC is the distance between the centers of the cell body and nucleus. AB represents the length of the cell radius and CD is the radius of the nucleus. Nuclear eccentricity of zero means that the center of the cell nucleus and cell body are identical, while 100% eccentricity refers to nuclei in contact with the cell body perimeter. Nuclear eccentricity was expressed as percentage (%) and used to classify neurons on a 3-point scale: type N, type I, and type II, based on previous observations (Gonzalez et al. 2004). "Normal" (type N) motoneurons presented a triangular shape, well-defined Nissl bodies, and nuclear eccentricity ranging between 0 and 33%. "Type I" motoneurons displayed some degree of nuclear eccentricity (33–66%), rounded shape and a tendency of Nissl bodies to accumulate at the periphery, whereas "type II" motoneurons exhibiting signs of advanced chromatolysis showed eccentric nuclei (66–100%) and finely dispersed Nissl material (Price and Porter 1972).

Nuclear eccentricity was studied in five ($n = 5$) animals per group. Double counting of neurons was unlikely because of the 50-μm spacing left between consecutive sections. In addition, the presence of a prominent nucleolus avoided counting the same cell twice. Analysis of the relative frequency distributions was performed with nonparametric statistics (χ^2 test) followed by partitioning analysis of contingency tables (Siegel and Castellan 1989).

MAP2 Immunocytochemistry

Antigenic sites for MAP-2 are present in the dendritic tree and neuronal cells bodies (Kikuchi et al. 1999; Yu et al. 2000). In our study, we analyzed MAP2 immunoreactivity in dendrites only. Forty micrometer free-floating sections obtained at the L1 level with a vibrating microtome were processed for MAP2 ICC. After treatment with H_20_2 in methanol (1:100), sections were exposed to a primary monoclonal anti-MAP2 (clone HM2, Sigma) recognizing the high molecular weight (280 kDa) forms of MAP2 (2a and 2b), but not the embryonic MAP2c isoform (Farah et al. 2005) and then reacted with a biotinylated anti-mouse IgG (Vector laboratories, Burlingame, CA, USA). Sections were further processed following the ABC kit instructions (Vector) and developed with 3,3′-diaminobenzidine (0.5 mg/ml; Sigma) in 0.05% H_2O_2. For negative controls, the primary antibody was omitted from the immunostaining procedure.

For semi-quantitative analysis of MAP2-immunopositive fiber density, we used previous reported protocols. The Optimas Program used in our analysis transforms differences in color intensity of immunopositive processes into gray differences (Gonzalez et al. 2004). The results were expressed as the inverse logarithm of gray intensity per area (ILIGV/area). Sections were mounted and processed simultaneously under identical operating conditions, such as light beam, wavelength, and gray-scale threshold throughout the experiment. Images were acquired at the same magnification using a digital camera Panasonic GP-KR222 connected to an Olympus BH2 microscope and the Image software Bioscan Optimas VI (Labombarda et al. 2003). After background substraction and gray-scale threshold determination, the area covered by MAP2-positive fibers was computed in the ventral horn of each section and expressed as MAP2-immunopositive fiber density (ILIGV/mm^2). Five sections from segment L1 caudal to the injured zone were analyzed in every animal (four animals per group). For statistical analysis, we applied one-way ANOVA, followed by post hoc comparisons with the Newman–Keuls test.

Results

Electron Microscopy

The ultrastructure of the spinal cord was compared in sham-operated, control rats, control + progesterone-treated rats, rats with complete spinal transection at thoracic level 10 (T10), and rats with spinal transection and progesterone treatment. Hormone-treated rats received four progesterone injections at 1, 24, 48, and 72 h post-injury, respectively. In sham-operated rats, motoneurons located of Rexed's Lamina IX showed the expected normal morphology (Nacimiento et al. 1995). Figure 1a shows the fine structure of a neuronal nucleus from a control rat. The nuclear chromatin displayed the typical euchromatic structure with granular appearance and small clumps of

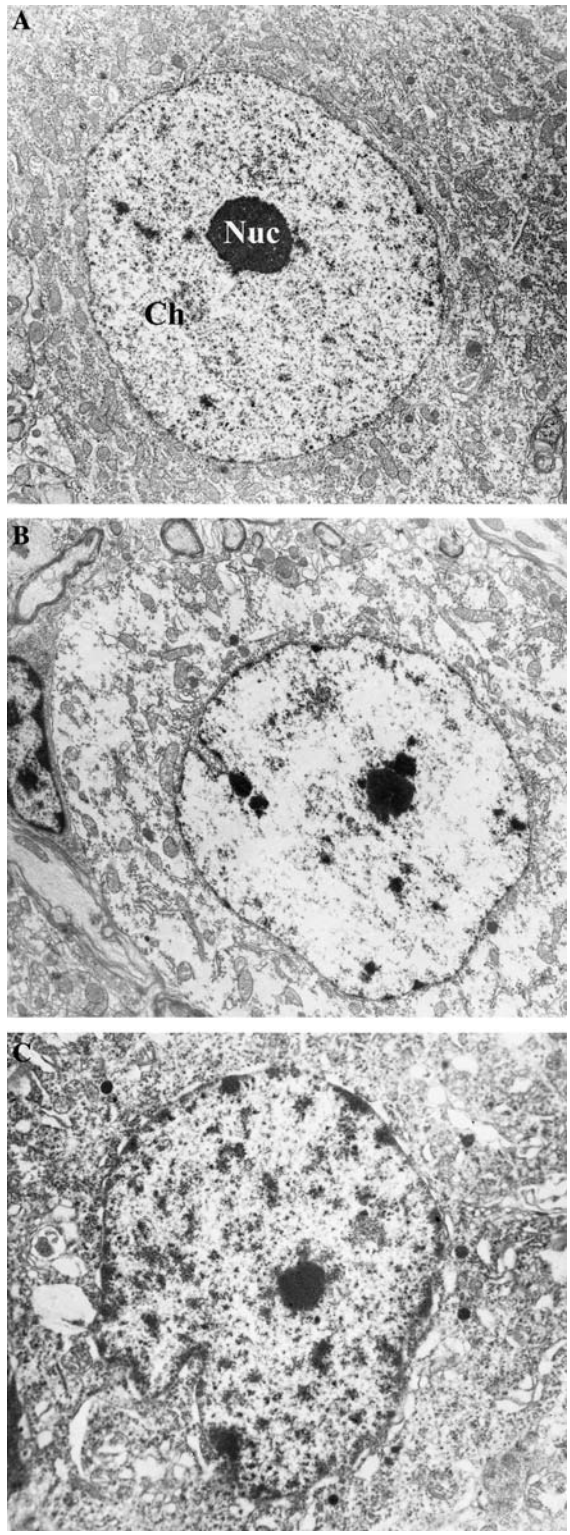

Fig. 1 (**a**) Nuclear ultrastructure in a motoneuron from a control sham-operated rat. This nucleus shows a compact nucleolus (Nuc) and in the nucleoplasm, the major part of chromatin (Ch) is euchromatic. Heterochromatin either attaches to the inner nuclear envelope or presents as minor and scattered clumps in the nucleoplasm. (**b**) Rat with spinal cord injury. Three days after complete spinal cord transection, the nucleus is more translucent with finely dispersed chromatin. Note that heterochromatin clumps are denser than in the control motoneuron. The fragmented ultrastructure of the surrounding cytoplasm is due to a disruption of the endoplasmic reticulum. (**c**) Nucleus from a motoneuron in the injured + progesterone groups. The chromatin appears denser with numerous heterochromatin clumps in the periphery as well as in the nucleoplasm. There is a partial recovery of the ultrastructure of the surrounding cytoplasm. Magnification 4,400×

heterochromatin attached to the nuclear envelope. The nucleolus appeared as a compact and electron dense spherical mass. In this control group, neuronal karyoplasm showed Nissl bodies composed of regular and parallel cisternae of rough endoplasmic reticulum (RER) with ribosomal particles attached to the outer surfaces of the cisternae and surrounded by vesicles and mitochondria (Fig. 2a). Motoneurons in the control + progesterone group presented an identical ultrastructural profile (data not shown), which is in agreement with biochemical studies showing the lack of hormonal effects in control animals (Gonzalez et al. 2005; Labombarda et al. 2003).

Electron microscopy also revealed that 3 days after spinal injury the nuclei of most neurons below the lesion site showed scattered chromatin, resulting in lower electron density (Fig. 1b). In contrast to the control group, injured motoneurons presented randomly arranged fragments of RER and disorganized cytoplasm with dissolution of Nissl bodies (Fig. 2b). In addition, the nucleus displayed an eccentric localization and the RER showed a substantial loss of the normal canalicular structure, as further illustrated in Fig. 3a, b. However, the 3-day course of progesterone treatment of injured rats strikingly modified these features. Thus, the nucleoplasm of neurons in this group appeared less translucent, showing instead increased electron density and condensed clumps of heterochromatin (Fig. 1c). Hormone treatment of injured animals also restored the amount and morphological distribution of Nissl substance, with preserved organization of the

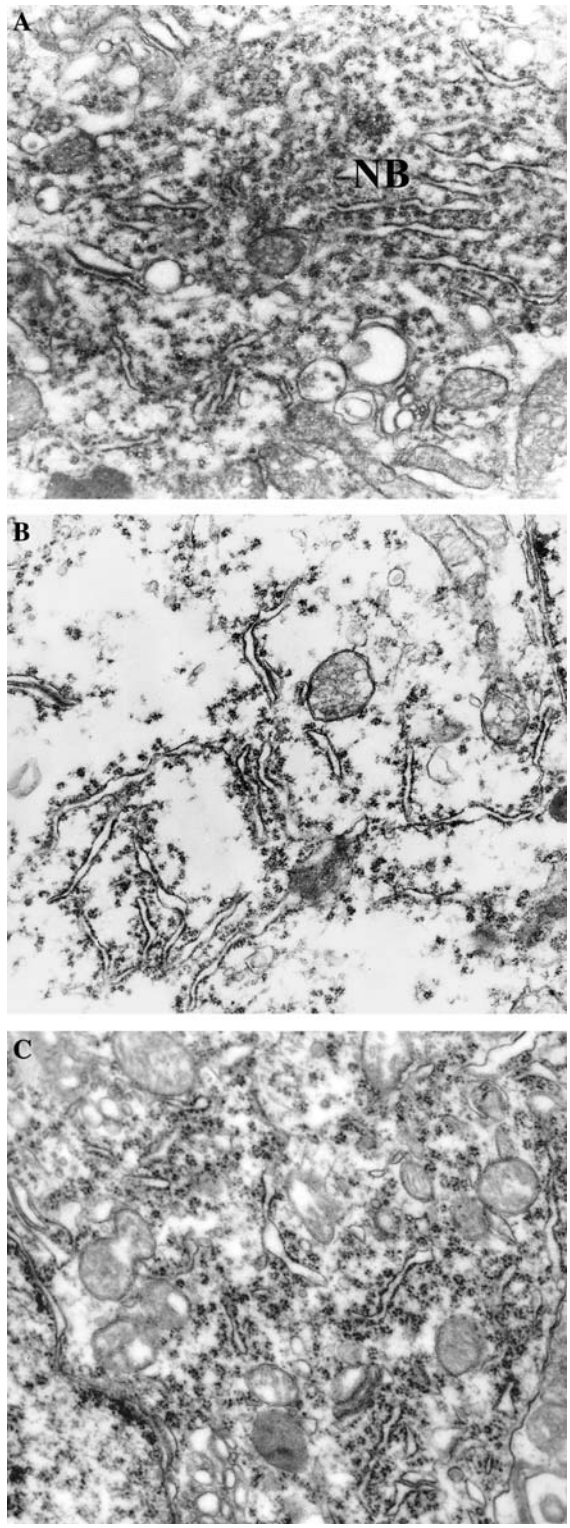

Fig. 2 (**a**) High magnification view of the cytoplasm in motoneuron from a control rat. The Nissl body (NB) is composed of regular and parallel cisternae of RER. (**b**) Cytoplasmic changes brought by spinal cord injury. The pattern suggests a chromatolytic-like reaction. Note the disorganized cisternae of RER that no longer aggregate in discrete Nissl bodies and the increased number of free polyribosomes. (**c**) Motoneuron in a spinal cord injured + progesterone-treated rat. The cytoplasm presented better-ordered cisternae of RER with attached ribosomes. Some degree of vacuolation persisted. Magnification 16,000×

cisternae of the RER and decreased number of free ribosomes (Fig. 2c). The ultrastructural appearance of nucleoli pointed out important differences between motoneurons from the control and injured groups (Fig. 4). A situation common to both the vehicle- or progesterone-treated injured rats but almost absent in control animals was the presence of light areas corresponding to the fibrilar portion in the central part of the nucleoli (Fig. 4b, c). These light areas may correspond to sites with high synthetic rate for ribonucleoproteins, developing in response to the depletion of Nissl bodies (Price and Porter 1972).

Nuclear Eccentricity

Since nuclear displacement is a typical sign of injured neurons, we analyze if steroid treatment normalized this parameter. For this analysis, motoneurons were classified as types N, I, or II, according to the presence of a normal, moderate, or severe eccentric nuclear phenotype. Figure 5a shows representative light micrographs of cresyl-violet stained motoneurons showing a predominance of neuronal bodies with severe eccentric nuclei and chromatolytic-like profiles in the injured animals as compared to the spinal cord injury animals + progesterone (Fig. 5a). Furthermore, nonparametric statistics demonstrated that significantly differences existed among experimental groups ($\chi^2 = 46.65$, $P < 0.0001$). The frequency histograms of Fig. 5b show that 75% of neurons in the control and control + progesterone groups presented a normal nuclear localization (type N, open columns). Following spinal cord injury, most of the injured cells showed either a mild (30%, stippled columns) or severe nuclear eccentricity (50%, dark columns), whereas only 20% remained normal ($P < 0.005$ vs. control and control + progesterone). In contrast, in the spinal cord

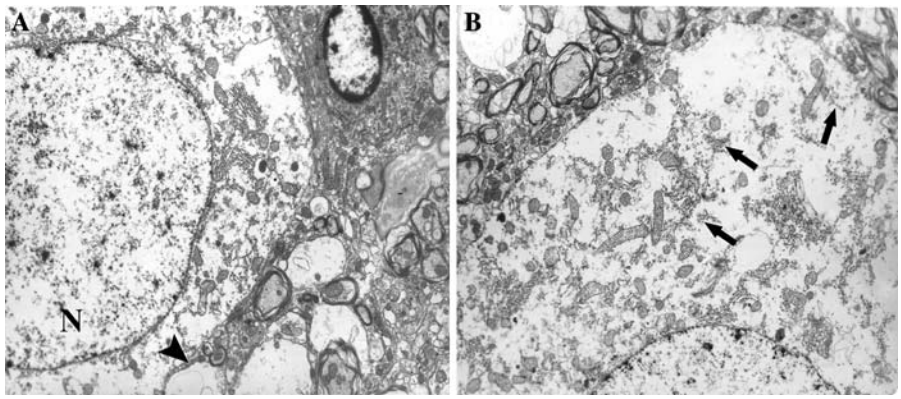

Fig. 3 Ultrastructural abnormalities after spinal cord injury, showing that typical signs of chromatolysis, are already present 3 days after the lesion. (**a**) Note the abnormal localization of the nucleus (N), displaced near the cell membrane (arrowhead). (**b**) Ribosomes dissociate from their membranes and disperse throughout the cytoplasmic matrix (arrows). Magnification 4,000×

injury + progesterone group, nuclei were located in a normal position in 64% ventral horn neurons, whereas just a few presented the mild (20%) or the severe phenotype (16%) ($P < 0.005$ vs. spinal cord injury group, Fig. 5b).

MAP2 Immunocytochemistry

In harmony with previous reports (Kikuchi et al. 1999; Yu et al. 2000), the spinal cord of control animals exhibited intense MAP2 immunostaining of dendrites and cell bodies (Fig. 6a). This profile was unchanged in the control + steroid-treated animals. In contrast, 3 days after spinal cord transection, MAP2 immunoreactivity dramatically decreased in both gray matter dendrites and cell bodies caudal to the injured site. This finding suggests a pronounced dendrite degeneration following spinal cord injury. In contrast, the spinal cord injury + progesterone group showed significantly increased staining with the MAP2 antibody as compared to the lesioned group. Semi-quantitative analysis of staining density of dendritic trees of gray matter (Fig. 6b) showed a significantly reduced immunoreaction labeling (expressed as ILIGV/area) in the spinal cord injury group (dark column) as compared to the control and control + progesterone groups (open and stippled columns, $P < 0.001$). However, steroid administration significantly enhanced the MAP2-immunopositive staining (spinal cord injury + progesterone, vertical line column) respect of the spinal cord injury group ($P < 0.001$).

Discussion

The present experiments demonstrated that within 3 days of spinal cord injury, progesterone modified in part ultrastructural abnormalities and preserved MAP2 immunostaining in deafferented motoneurons. These results bring a morphological substrate to previous observations showing progesterone effects in the injured spinal cord (Gonzalez et al. 2004; Labombarda et al. 2002, 2003). Although the spinal cord can synthesize its own neurosteroids (di Michele et al. 2000; Labombarda et al. 2006b; Patte-Mensah et al. 2004), supplementary progesterone dosage may be required to produce the above-mentioned effects.

After complete spinal transection, changes developed in the nucleus, karyoplasm, and nucleolus. The nucleus of lesioned motoneurons displayed reduced electron density, scattered chromatin, and adopted an eccentric position, accompanied by chromatolytic changes in the cytoplasm, such as fragmented RER, dissolution of Nissl bodies, and vacuolation. However, the absence of plasma and nuclear membrane blebbing, apoptotic bodies, and pyknotic nuclei fit better with a form of cell death called paraptosis rather than classical apoptosis (Leist and Jaattela 2001; Kaur et al. 2007; Sperandio et al. 2000). It is possible that the time schedule employed to analyze the effects of spinal cord injury or the use of animals with complete spinal cord transection could have conditioned the reported neuropathology. Signals for chromatolytic-like alterations are not fully understood (Cragg 1970). A disorganized

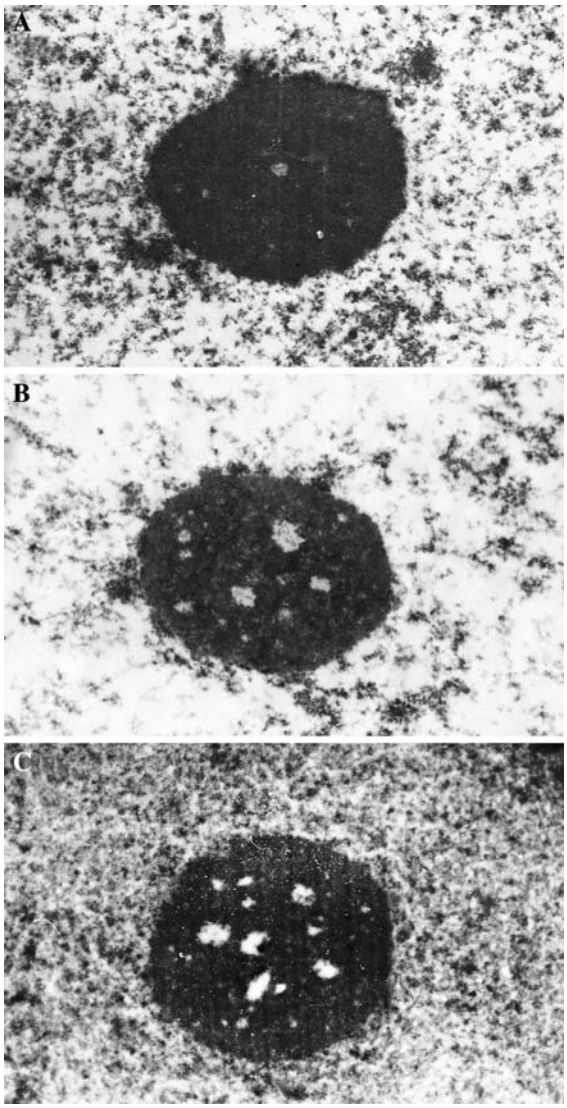

Fig. 4 Comparison of nucleolar ultrastructure in a normal motoneuron (**a**), a motoneuron 3 days after complete spinal transection (**b**) and a motoneuron from a transected animal receiving progesterone treatment (**c**). Note the extensive fibrilar areas of the nucleolus in (**c**) and (**b**), indicating nucleolar hyperactivity. Magnification 20,000×

Nissl substance, comprising membranes, endoplasmic reticulum, and ribosomes, occurs under pathological conditions like axotomy, amyotrophic lateral sclerosis, and spinal injuries (Gonzalez et al. 2004; Nacimiento et al. 1995; Price and Porter 1972; Wakayama 1992), and represents the pathological landmark of the neuronal reaction to injury. In addition, the increased incidence of eccentric nuclei in large spinal motoneurons suggested that injury had widespread consequences for neuronal function.

Ultrastructural changes also occur in the nucleolus, the site of synthesis of the ribosomes, which are the basophilic component of Nissl substance. Electron microscopy observations in the injured rats showed that the nucleolus presented small light areas, possibly related to the high synthetic activity of this structure (Price and Porter 1972). In this respect, the centrifugal disappearance of Nissl bodies seems to stimulate the nucleolus to elaborate extra material for replacement of the depleted ribonucleoproteins. Interestingly, in progesterone-treated rats, nuclear morphology was intermediate between the normal pattern and that found in injured animals, suggesting a partial recovery. Second, the nucleolus of both hormone naïve and hormone-treated animals showed morphological changes in the fibrilar portion, which in the case of progesterone-receiving rats, accompanied cytoplasmic replenishment of Nissl bodies. This finding suggests a more efficient synthesis of ribonucleoproteins in hormone-repleted animals. Third, progesterone also modified the nuclear eccentricity showed by injured motoneurons. In this case, a large percentage of the motoneurons showed a centrally located nucleus resembling normal motoneurons, in contrast to the injured group in which at least half of the motoneurons showed nuclear eccentricity. A suggested mechanism for the development of nuclear eccentricity includes the interference with axonal transport and accumulation of axonal components in the injured cell body (McIlwain and Hoke 2005). We have not explored whether there is axonal impairment in this model of spinal cord injury. However, recent evidences in another model suggest that progesterone may correct slow axonal transport. The latter is impaired in mice with spinal cord neurodegeneration, but recovers after progesterone treatment (Gonzalez Deniselle et al. 2005). In consonance with findings in degenerating motoneurons, it is possible that after spinal cord injury, progesterone facilitated axonal transport and avoided the accumulation of components causing nuclear displacement.

Several studies have emphasized that axonal lesions deprive cells of neurotrophic factors trigger the chromatolytic reaction and lead to an eccentric localization of the nucleus (Gold et al. 1991). In addition to trans-neuronal signals (Nacimiento et al. 1995; Eidelberg et al. 1989) depletion of endogenous neurotrophic factors after injury, in particular brain-derived

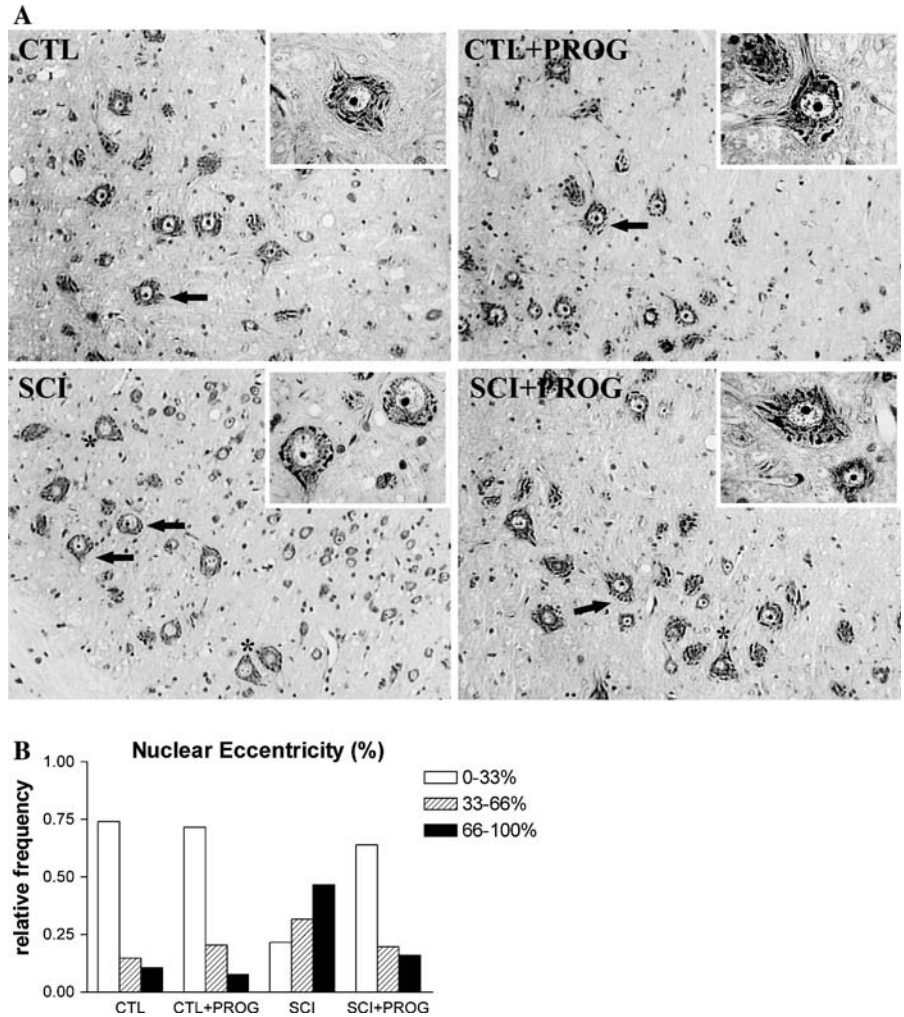

Fig. 5 (**a**) Evaluation of nuclear eccentricity in cresyl-violet stained motoneurons from the four experimental groups (five animals per group). Light microphotographs denote the predominance of neuronal bodies presenting severe eccentric nuclei (asterisks) in a rat after spinal cord injury (SCI) as compared to a SCI + progesterone-treated (SCI + PROG) animal. Arrows point to motoneurons enlarged in the insets. Insets: high-power views of normal-appearing cells (insets in CTL, control; CTL + PROG, control + progesterone; SCI + PROG, spinal cord injury + progesterone) and two cell bodies with eccentric nuclei from a lesioned animal (inset in SCI). Magnification, 40×; insets, 100×. (**b**) Frequency histograms showing percent (%) nuclear eccentricity in neurons from the CTL ± PROG and SCI ± PROG groups, as determined by the method of Barr and Hamilton (1948). We classified neurons as type N (normal, 0–33% NE), type I (mild chromatolytic, 33–66% NE), and type II (severe chromatolytic, 66–100% NE). Analysis of the relative frequency distributions was performed with nonparametric statistics (χ^2 test) followed by partitioning analysis of contingency tables ($\chi^2 = 46.65$, $P < 0.0001$). Three days after SCI, 50% of motoneurons presented a severe eccentric localization of the nuclei. This distribution was significantly different from CTL and CTL groups ($P < 0.005$ vs. CTL and CTL + PROG). In contrast, the SCI plus progesterone group presented the normal position of the nuclei in 64% of neurons and only 16% presented the severe phenotype ($P < 0.005$ vs. SCI)

neurotrophic factor, may lay behind the structural abnormalities of motoneurons leading to cell dysfunction and neurodegeneration. The involvement of neurotrophic factors is of outmost importance to understand the present findings. In this context, it is relevant that progesterone significantly enhances brain-derived neurotrophic factor expression after injury (González et al. 2005) in neurodegeneration of the spinal cord and in cortical explants in culture (Gonzalez Deniselle et al. 2007; Kaur et al. 2007). It

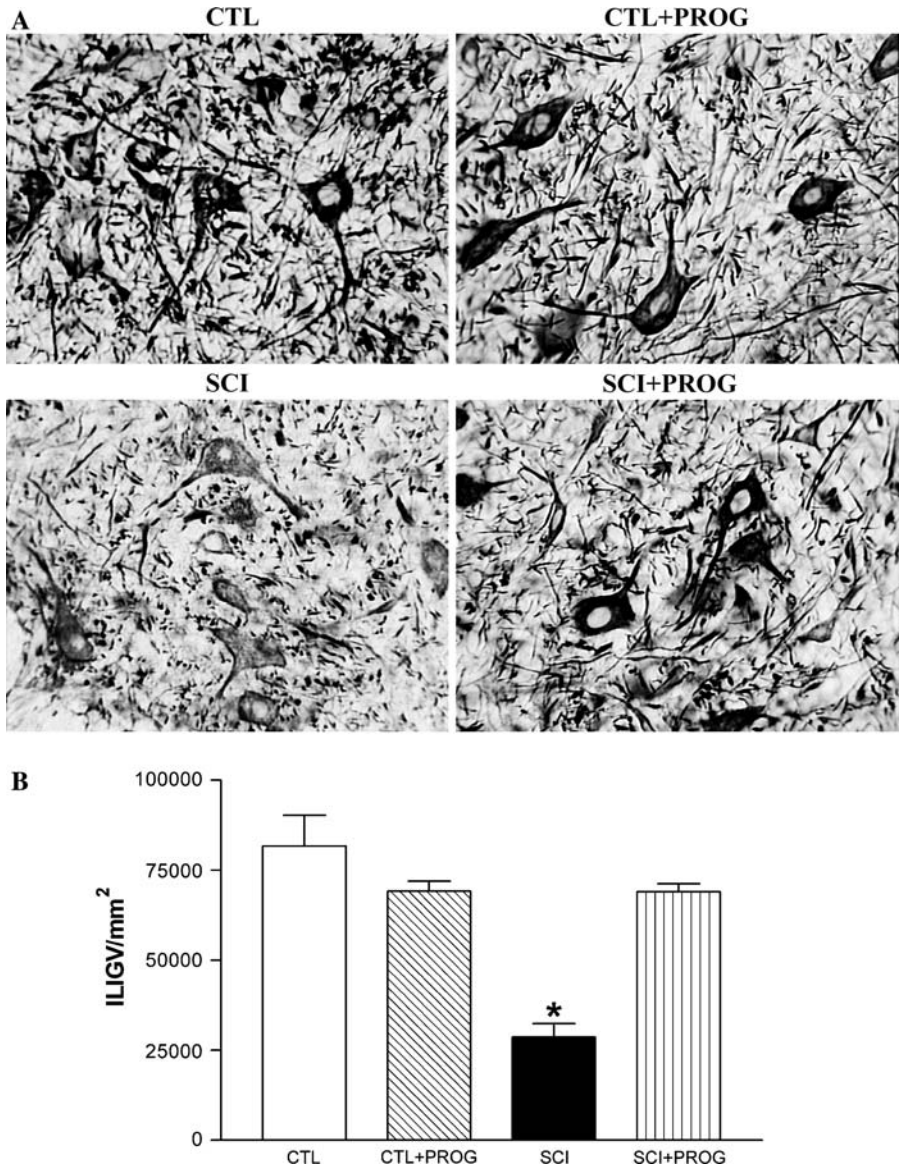

Fig. 6 Effects of SCI and steroid treatment on MAP2 immunoreactivity. (**a**) Strong MAP2 immunostaining of gray matter dendrites and cell bodies characterized the control ± progesterone (CTL, CTL + PROG) groups. Three days after SCI, injured animals receiving vehicle show weak staining of dendrites and cell bodies, in contrast to the strong MAP2 staining of these structures in steroid-receiving injured animals (SCI + PROG). (**b**) Quantification of MAP2 staining intensity (ILIGV/mm^2, mean ± SEM). The immunoreaction intensity is greatly attenuated in the SCI group receiving vehicle (dark column) as compared to CTL and SCI groups receiving progesterone (open, stippled, and vertical line columns, $P < 0.001$). We analyzed five sections from segment L1 caudal to the injured zone in every animal (four animals per group) by one-way ANOVA, followed by the Newman–Keuls test

has been hypothesized that the locally synthesized brain-derived neurotrophic factor may act as an autocrine/paracrine regulator of neuronal functions (Acheson and Lindsay 1996; Davies 1996; Miranda et al. 1993). In addition to brain-derived neurotrophic factor, progesterone is an important immunomodulator, opposing the inflammatory response (Grossman et al. 2004; Stein 2005). Although we did not study the mechanism of action of progesterone in spinal cord injury, a recent study demonstrated that in rats with traumatic brain injury, progesterone decreases the expression of COX-2, IL-6, and NFkappaB (Cutler et al. 2007). This suggests that anti-inflammatory effects are mechanistically important for progesterone neuroprotection. Thus, the present study needs additional analysis to see whether progesterone treatment would be associated to changes in inflammatory mediators. In a mouse model of multiple sclerosis, progesterone decreases reduces the infiltration of inflammatory cells in the spinal cord (Garay et al. 2007), suggesting anti-inflammatory actions.

As expected from literature reports (Springer et al. 1997; Yu et al. 2000; Zhang et al. 2000), spinal cord injury brought about changes of MAP2 staining. A loss of MAP2 immunoreactivity also occurs in a variety of pathological conditions exposed to excess

calcium influx and calpain activation, NMDA-activation, oxidative stress, and dephosphorylation (Farah et al. 2005; Kikuchi et al. 1999; Yu et al. 2000). Progesterone treatment of rats with spinal injury also unregulated MAP2 staining in dendrites and perikaryon, suggesting effects on the cytoskeleton (Di Stefano et al. 2006). In circumstances other than spinal injury, progesterone also enhances MAP2 protein expression. For instance, female rats show less pronounced trauma-induced cytoskeletal damage by calpain, caspase-3, and calcium overload than male rats (Kupina et al. 2003). In hippocampus, cerebellum, and PC12 cells, neuroprotection by sex steroids involves changes of cytoskeleton organization, binding of neurosteroids to the MAP2 molecule and control of cytoskeleton dynamics (Fontaine-Lenoir et al. 2006; Ghoumari et al. 2003; Reyna-Neyra et al. 2002). In spite of the fact that the spinal motoneurons express classical PR (Labombarda et al. 2003), PR response elements are absent from the MAP2 gene. Supporting this notion, progesterone increases MAP2 protein but not mRNA in hippocampus, suggesting a post-transcriptional effect. (Reyna-Neyra et al. 2002). In mature neurons, distribution and content of MAP2 are regulated transsynaptically, suggesting that progesterone and/or its reduced metabolites could regulate MAP2 by mechanisms involving inhibitory and excitatory neurotransmission (Caceres et al. 1988).

Of further interest is the notion that MAP2 immunoreactivity is associated with polyribosomes and that MAP2 may play a role in RER membrane positioning (Bernhardt and Matus 1984; Farah et al. 2005). Our study showed that the injury-induced loss of MAP2 and the disruption of RER membranes were modulated by steroid administration. Furthermore, cytoskeletal proteins are downstream targets of neurotrophins, which recover the cytoskeleton and protect its proteins from proteolytic degradation (reviewed in Hayes et al. 1995). Brain-derived neurotrophic factor positively modulates the expression and phosphorylation state of MAP2 (Fukumitsu et al. 1997; Marsh et al. 1993). These findings further suggest that this neurotrophin may be one of the factors mediating progesterone effects on the cytoskeleton and on development of the chromatolytic reaction.

In conclusion, exogenous progesterone normalized in part some ultrastructural abnormalities and increased MAP2 expression in motoneurons from rats with acute spinal cord injury. Further studies will ascertain if progesterone effects are maintained in rats with long-term spinal cord injury, an objective requiring further neurochemical, morphological, and functional studies.

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