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Effects of progesterone in the spinal cord of a mouse model of multiple sclerosis

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Abstract

The spinal cord is a target of progesterone (PROG), as demonstrated by the expression of intracellular and membrane PROG receptors and by its myelinating and neuroprotective effects in trauma and neurodegeneration. Here we studied PROG effects in mice with experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis characterized by demyelination and immune cell infiltration in the spinal cord. Female C57BL/6 mice were immunized with a myelin oligodendrocyte glycoprotein peptide ($MOG_{40–54}$). One week before EAE induction, mice received single pellets of PROG weighing either 20 or 100 mg or remained free of steroid treatment. On average, mice developed clinical signs of EAE 9–10 days following MOG administration. The spinal cord white matter of EAE mice showed inflammatory cell infiltration and circumscribed demyelinating areas, demonstrated by reductions of luxol fast blue (LFB) staining, myelin basic protein (MBP) and proteolipid protein (PLP) immunoreactivity (IR) and PLP mRNA expression. In motoneurons, EAE reduced the expression of the alpha 3 subunit of Na,K-ATPase mRNA. In contrast, EAE mice receiving PROG showed less inflammatory cell infiltration, recovery of myelin proteins and normal grain density of neuronal Na,K-ATPase mRNA. Clinically, PROG produced a moderate delay of disease onset and reduced the clinical scores. Thus, PROG attenuated disease severity, and reduced the inflammatory response and the occurrence of demyelination in the spinal cord during the acute phase of EAE.

Keywords: EAE; Multiple sclerosis; Progesterone; Myelination; Neuroprotection

1. Introduction

Progesterone (PROG) is a recognized promyelinating and neuroprotective hormone for nervous system diseases. Thus, PROG preserves neurons following brain contusion, ischemia and section of the hypoglossal and facial motor nuclei [1–3]. In the spinal cord, PROG increases motoneuron survival after axotomy or injury, protects cultured neurons against glutamate toxicity and normalizes defective functional parameters of injured neurons [4–6]. In Wobbler mouse motoneurodegeneration, PROG slows the development of the disease, increases survival, axonal transport, the expression of neurotrophic factors and decreases oxidative stress [7,8].

In conjunction with neuronal effects, PROG strongly influences myelin synthesis in the peripheral and central nervous system [9–11]. In the oligodendrocytes, the central myelin-producing glia, PROG increases myelination in culture and in cerebellum, as shown by the increased expression of myelin basic protein (MBP) [10–12]. A similar effect on myelination takes place in the lesioned spinal cord when rats receive systemic PROG treatment [13]. PROG also increases the proliferation and differentiation of oligodendrocyte precursor cells that play an important role in remyelination after toxin-induced lesions and aging [11,12]. Additionally, PROG receptors are found in spinal cord motoneurons and oligodendrocytes and PROG may contribute to oligodendro-

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cyte progenitor proliferation [13–15]. In addition to neuronal and myelinating effects, PROG also modulates the immune system, changing a Th1 pro-inflammatory response to a Th2 anti-inflammatory response [16–19]. In traumatic brain injury, PROG decreases the surge of the inflammatory factors NFκB p65, complement factor 3, TGFβ2 and IL-1β [20,21].

To study the myelinating and immunomodulatory properties of PROG, we used mice with experimental autoimmune encephalomyelitis (EAE), an induced immune inflammatory disease of the central nervous system (CNS) which resembles human multiple sclerosis (MS) [22–24]. EAE may be induced with a number of myelin proteins, including myelin oligodendrocyte glycoprotein (MOG). MOG is expressed on the outer surface of oligodendrocytes and its administration has many features in common with MS [25]. Rodents with EAE present spinal cord demyelination, inflammatory cell infiltration, microglial activation, axonal loss, astrocytosis, proliferation of oligodendrocyte precursor cells and neuronal dysfunction [26–29]. Clinically, afflicted mice show typical neurological deficits, ranging from loss of tail tonicity to several degrees of paralysis to death [25,26,30].

A variety of agents have been employed to alleviate EAE based on their immunomodulatory and anti-inflammatory properties [29,31–35]. Glucocorticoids, for instance, are potent immunosupressors and prevent EAE [36], whereas estrogens show neuroprotective effects and ameliorate EAE by a mechanism involving estrogen receptor alpha (ERα) [37–41]. Pregnancy has a beneficial effect for MS patients, slowing the rate of progression and disability whereas disease worsening reappears after delivery. It has been proposed that the increased levels of circulating PROG at the time of pregnancy may afford protection against MS [17,42]. However, PROG have variable effects in EAE, ranging from inactivity, increased vulnerability of neurons, to disease improvement when given with estradiol [42–44].

In this study, we analyzed the effects of PROG on spinal cord neuropathology and clinical outcome of EAE. We studied if PROG administration beforehand attenuates spinal cord neuropathology, following a treatment protocol in which animals pretreated with estrogen are less prone to subsequent EAE induction [37,38,40,44]. To investigate PROG effects on myelin, we studied the myelin proteins MBP and proteolipid protein (PLP), whereas effects on motoneurons was analyzed by determination of neuronal Na,K-ATPase mRNA. Correspondingly, the effects of PROG were also assessed on clinical disease scores of EAE mice.

2. Materials and methods

2.1. Experimental animals

C57BL/6 female mice (9–11 weeks old) remained untreated or received sc a single 20 mg or 100 mg PROG pellet (Sigma Chem. Co, St. Louis, MO) one week before EAE induction. EAE was induced using a peptide corresponding to the published sequence of rodent myelin oligodendrocyte glycoprotein $MOG_{40–54}$ (YRSPFSRVVHLYRNG) [30]. Originally, this peptide was kindly donated by Dr. D. Sun [30] and later batches were synthesized by Dr. Clara Peña (Faculty of Pharmacy and Biochemistry, University of Buenos Aires) [45]. Experimental mice received a sc injection on each flank of 200 μg $MOG_{40–54}$ per mouse emulsified in complete Freund's adjuvant CFA (Sigma) containing 0.6 mg *Mycobacterium tuberculosis* (Instituto Malbran, Argentina). The animals received injections of *Pertussis* toxin ip (400 ng) (Sigma) immediately after immunization and another boost on the day after. Some animals received CFA and *Pertussis* toxin without MOG but none of them developed signs of EAE. Mice were monitored daily for weight loss and neurological signs of EAE. Disease severity was scored as previously published for EAE mice [25,30]: grade 0 = no signs, grade 1 = partial loss of tail tonicity, grade 2 = loss of tail tonicity, difficulty in righting, grade 3 = unsteady gait and mild paralysis, grade 4 = hind-limb paralysis and incontinence, grade 5 = moribund or death. EAE in hormone-free animals developed approximately on day 10 and they were sacrificed on day 16 when the disease was still in an acute phase.

Three clinical parameters were analyzed regarding development of EAE: (a) EAE severity, calculated as the disease index (DI) for each group of mice. DI represented the mean of the daily clinical score for all mice in the group divided by the mean day of onset, multiplied by 100 [46], (b) disease onset, calculated by averaging the first day of clinical signs for each mouse in the group, and (c) the peak disease score, similarly calculated by averaging the highest individual score for each mouse in the group. Animal procedures followed the Guide for the Care and Use of Laboratory Animals (NIH Guide, Institute's Assurance Certificate # A5072-01) and were approved by the Institute's Animal Care and Use Committee.

2.2. Tissue preparation

Animals were deeply anesthetized with ketamine (50 mg/100 g BW) and transcardially perfused with 0.9% NaCl prepared in diethylpirocarbonate-treated water, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. Cervical and lumbar spinal cords were carefully removed, postfixed in the same fixative for 2.5 h at 4 °C and processed for routine paraffin embedding. Other batches of spinal cords were cryoprotected by immersion in 20% sucrose overnight, and kept frozen at −70 °C until used. Paraffin sections were stained with haematoxylin and eosin (H&E) to asses immune cell infiltration and with luxol fast blue to mark the area of demyelination. LFB staining was carried out according to Kim et al. [47]. The LFB negative area, representating white matter demyelination, was determined and expressed as described below for MBP and PLP immunostaining.

2.3. Immunohistochemistry for detection of myelin proteins

Five micrometer sections of paraffin-embedded spinal cords were deparaffinized and treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase. Immunocytochemistry was carried out as described before [4] using a 1:500 dilution of rabbit anti-MBP primary antibody (Dako, Cytomation) or a 1:100 dilution of the AA3 rat anti-PLP antibody (kindly provided by Dr. S.R. Winkler, Department of Neuroscience, University of Connecticut School of Medicine, Farmington, CT). Areas showing negative immunostaining for MPB or PLP were delimited at several regions of the spinal cord (dorsal, lateral or ventral funiculus) by computerized image analysis using Optimas VI software [13,48]. Surface areas of these regions were added up and demyelination for each spinal cord section was expressed as a percentage of the total surface area of white matter sampled. [28,49].

2.4. In situ hybridization for PLP and Na,K-ATPase mRNA

In situ hybridization (ISH) for PLP mRNA and the alpha 3 subunit Na,K-ATPase mRNA was carried out in cryostat sections following previously published protocols from our laboratory [7,50]. Coding sequences for PLP mRNA was 5′-TGTACCAGTGAGAGCTTCATGTCCACATCCACAGA-ACAGTGCCACTCC-3′ [51] and for the alpha 3 of the Na,K-ATPase subunit was 5′-CGGGAAGAGCGGCGGAC-AGGCTGGTGAGCGGTGGCCGCAGA-3′ [52]. Synthesis of oligonucleotides was carried out by Oligos Etc, Inc. (Wilsonville, OR). The PLP hybridization signal was found in glial cells only, whereas the Na,K-ATPase probe produced a strong signal in ventral horn motoneurons without labeling of glial cells.

For analysis of Na,K-ATPase mRNA, the number of grains in motor neurons was calculated after background subtraction. Quantitative grain counting was performed by computer-assisted image analysis equipped with a video camera (Bioscan Optimas VI). For Na,K-ATPase alpha 3 subunit mRNA, data from about 40 cells contained in both ventral horns per animal corresponding to four sections ($n = 6$ mice per group) were combined to give an animal mean, and the animals used as independent variables. Data were reported as number of grains/1000 μm^2 (mean ± SEM). For PLP mRNA, grains were counted in single cells of the gray matter corresponding to three sections per animal ($n = 4$–6 mice per group) and results expressed as the mean number of grains per oligodendrocyte.

2.5. Progestin levels in serum

The content of serum progestins was determined using a Coat-A-Count Progesterone RIA Kit (Diagnostic Product Corporation, Los Angeles, USA) and results expressed as ng/ml serum. Intra- and interassay coefficient of variation were 3.6 and 5.6%, respectively. Due to the relative specificity of RIA methods, the possibility existed of cross-reactions with PROG metabolites in hormone-treated mice. Thus, steroid levels were reported as serum progestins (ng/ml).

2.6. Statistical analysis

Differences in disease onset, disease index and peak disease score were analyzed by Student's *t* test, following Bebo et al. [37] and Palaszynski et al. [53] who used a similar statistical method for comparison. Significance was set at $p < 0.05$. Group differences for LFB, MBP/PLP immunostaining and *in situ* hybridization for PLP and Na,K^-ATPase mRNA were determined by one-way ANOVA, followed by post-hoc comparisons with the Newman–Keuls test.

3. Results

3.1. Progesterone reduced clinical severity of EAE in mice

PROG pellet implantation modified neurological deficits and clinical scores of EAE mice. As shown in Table 1, disease onset, which in hormone-untreated mice averaged ≈9.9 days, was significantly delayed by implantation of 20 and 100 mg PROG pellets. Both peak score and disease index, as defined in Section 2, were significantly reduced in EAE mice under both PROG doses. Serum progestins were low in hormone-free EAE mice (4.4 ± 2.1 ng/ml), increased 10-fold in animals receiving the 20 mg PROG pellet (39.5 ± 7.4, $p < 0.05$ versus EAE) and further increased 20-fold in mice bearing the 100 mg PROG pellet (86.5 ± 10.6, $p < 0.001$ versus EAE). The last group, in addition, contained significantly higher levels than those in the 20 mg PROG pellet group ($p < 0.01$).

Table 1
Effects of progesterone on neurological parameters of EAE mice

Animal group	Disease onset (days)	Peak score	Disease index
EAE	9.9 ± 0.27	4.0 ± 0.17	181.1 ± 13.4
EAE + PROG 20 mg	10.9 ± 0.34[b]	3.0 ± 0.44[a]	121.5 ± 20.9[a]
EAE + PROG 100 mg	12.0 ± 0.75[c]	3.1 ± 0.35[a]	100.0 ± 17.4[d]

EAE mice received a single 20 mg or 100 mg PROG pellet one week before EAE induction with the $MOG_{40–54}$ peptide. Disease onset was calculated by averaging the first day of clinical signs for each mouse in the group. Disease index was the mean of the daily clinical score of each animal in the group divided by the mean day of onset X 100, whereas peak score was calculated by averaging the highest individual score for each mouse in the group. Statistical analysis demonstrated: [a]$p < 0.05$ vs. EAE; [b]$p < 0.02$ vs. EAE; [c]$p < 0.002$ vs. EAE; [d]$p < 0.01$ vs. EAE. Number of animals for Disease Onset was EAE: 23; EAE + PROG 20 mg: 21 and EAE + PROG 100 mg: 10. For peak score and disease index, 9 animals per group were studied.

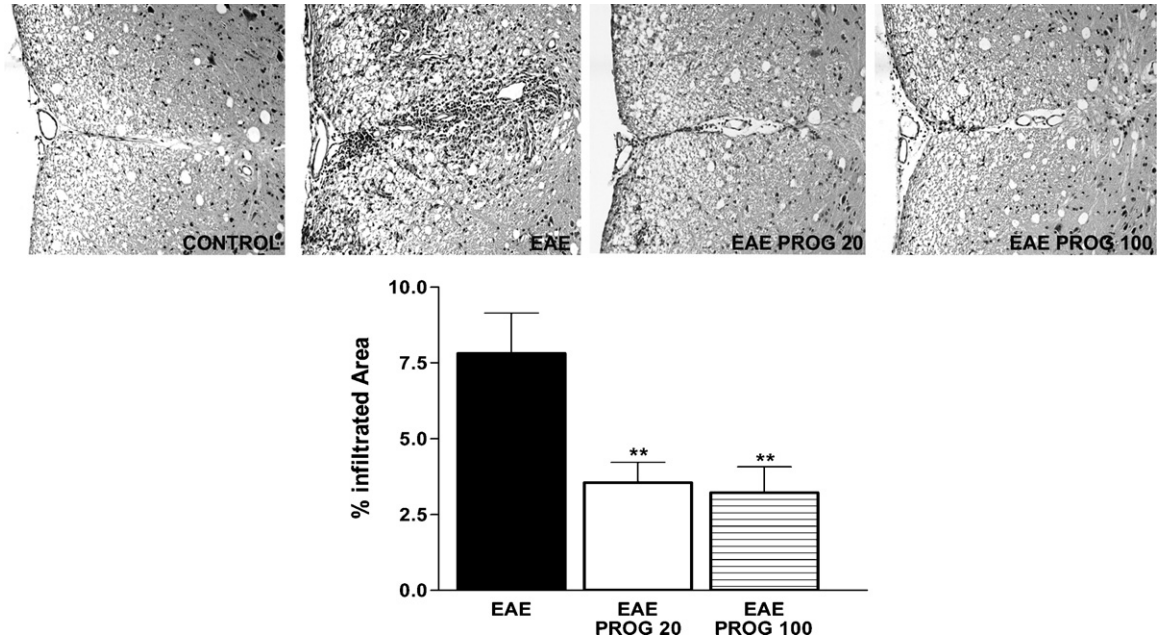

Fig. 1. Effects of EAE and progesterone (PROG) treatment on infiltration of inflammatory cells in the spinal cord. Spinal cord sections taken at the cervical level were stained with haematoxylin & eosin, and area of infiltration determined by computerized image analysis as described under Section 2. Upper photomicrographs show a control mouse, intense infiltration of the dorsal funicular area in EAE and its recovery after treatment with 20 or 100 mg PROG pellets. Lower graph: percent infiltration area in EAE was significantly decreased by PROG 20 and 100 mg pellets ($^{**}p < 0.01$ for both doses; $n = 8$–10 mice per group).

3.2. Progesterone reduced inflammatory cell infiltration and demyelinating lesions

In agreement with previous observations, the spinal cord of EAE mice contained inflammatory cells in several regions. The strongest infiltration of mononuclear cells was observed in the ventral and dorsal funicular white matter, which lies between the dorsal horns. The majority of these cells presented an ED1 phenotype characteristic of macrophages (data not shown). The profile of infiltrating cells in control mice, EAE, PROG 20 mg and PROG 100 mg-treated mice is shown in the photomicrographs of Fig. 1 (upper graph) which represents, from left to right a control, EAE, EAE + PROG 20 mg and EAE + PROG 100 mg treated mice. Quantitative analysis of cell infiltration in the experimental groups is shown in the lower graph of Fig. 1. The area covered by inflammatory cells, which measured 7.8% of the total white matter area in untreated EAE mice, was significantly reduced by the low and high PROG doses ($p < 0.01$ in both cases). Furthermore, LFB staining for total myelin demonstrated demyelinated lesions in circumscribed areas of the spinal cord of untreated EAE mice. This is shown in the left panels of Fig. 2, which represents, from top to bottom, LFB staining in control, EAE, EAE + PROG 20 mg and EAE + PROG 100 mg treated mice, respectively. Interestingly, percentage of demyelination, according to the absence of LFB reaction, was positively correlated with the area of inflammation ($r = 0.99$, $p = 0.0019$). The LFB unstained area (Fig. 2, arrows in EAE under LFB column) amounted to 8.2% of the total white matter area in EAE mice (Fig. 3, left hand graph). In mice receiving both PROG doses, LFB negative foci mostly disappeared (Fig. 2). Quantitative analysis of this variable (Fig. 3) showed that percentage demyelination was decreased by half in hormone treated mice, respect of steroid-naïve EAE mice (Fig. 3, left-hand graph; $p < 0.05$ for both PROG 20 mg and 100 mg, respectively).

3.3. PROG enhanced the expression of myelin proteins in EAE mice

Immunohistochemistry for MBP and PLP, the two major central myelin proteins, was also employed, in addition to LFB staining, to assess spinal cord demyelination. EAE-induced loss of myelin was predominantly observed in foci localized to the dorsal and ventral funiculus, although it also occurred in the ventrolateral white matter tracts in most affected animals. MBP and PLP immunoreaction images of the dorsal and ventral white matter tracts (Fig. 2, middle-panel under MBP and right-hand panel under PLP), showed negative immunoreactive MBP and PLP foci in untreated EAE mice (Fig. 2, arrows in EAE photomicrographs under MBP and PLP columns) respect of control and both groups of PROG-treated EAE mice (Fig. 2, EAE + PROG 20 mg and EAE + PROG 100 mg). Areas lacking MBP and PLP immunostaining respect of total spinal cord white matter immunoreactive area were 5.2% and 6.0% for MBP and PLP, respectively (Fig. 3, middle and right-hand graphs). These figures were significantly reduced by PROG 20 and 100 mg pellet implantation ($p < 0.05$ in both cases). The enhancement of myelin protein expression by PROG was similar for

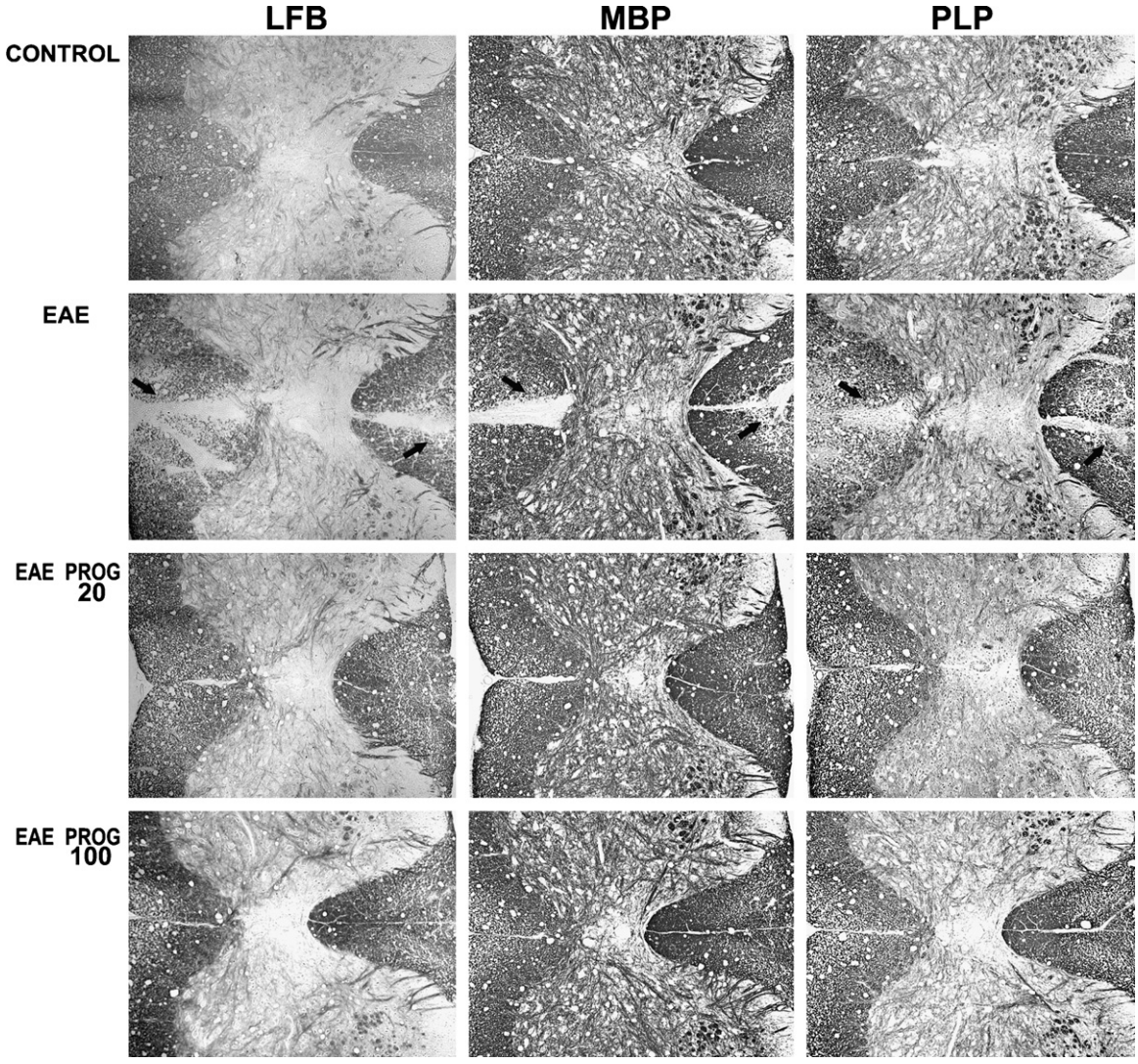

Fig. 2. Representative photomicrographs of luxol fast blue staining (LFB), immunoreaction for myelin basic protein (MBP) and proteolipid protein (PLP) in – from top to bottom – control, EAE, EAE + PROG 20 mg and EAE + PROG 100 mg-treated mice. Arrows in the EAE groups correspond to demyelinated areas free of LFB staining or MBP and PLP immunoreactivity. As can be seen in this figure, both doses of PROG were able to recover LFB staining as well as MBP and PLP immunoreaction intensity of EAE mice.

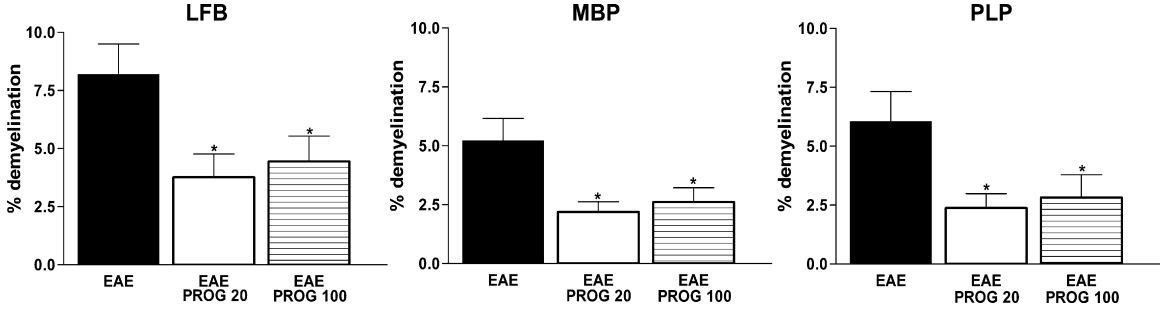

Fig. 3. Quantitative data for % demyelination respect of total white matter area according to luxol fast blue staining (LFB), immunoreaction for myelin basic protein (MBP) or proteolipid protein (PLP). The three graphs represent EAE (dark columns), EAE + PROG 20 mg (white columns) and EAE + PROG 100 mg (horizontal line columns). $^{*}p < 0.05$ vs. EAE ($n = 8$–10 mice per group).

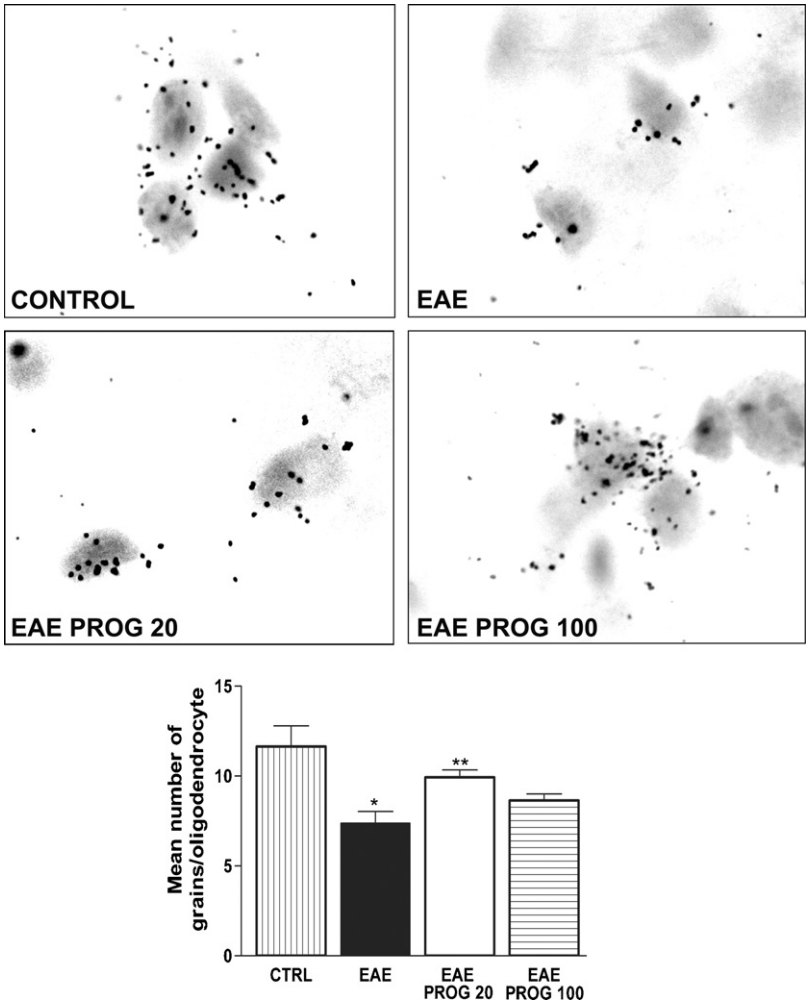

Fig. 4. *In situ* hybridization for PLP mRNA. The upper photomicrographs show grains over glial cells in a control mouse, grain depletion in EAE, grain repletion in EAE + PROG 20 mg and partial repletion in EAE + PROG 100 mg. Grain counting (lower graph) confirmed that PLP mRNA was lower in EAE vs. control (CTL) ($^{*}p < 0.01$) and EAE vs. EAE + PROG 20 mg ($^{**}p < 0.01$) but not in EAE vs. EAE + PROG 100 mg treated mice. Data were gathered from grain-containing single cells visualized in three different spinal cord sections per mouse ($n = 4$–6 mice per group).

MBP and PLP, and was maximally observed in the dorsal and ventral funiculus of the spinal cord.

Myelin recovery following PROG treatment was also studied at the PLP mRNA level. In this experiment, accumulated grains due to probe hybridized to PLP mRNA were observed over small, presumably oligodendroglial cells populating the gray matter and the white matter of the spinal cord (Fig. 4, photomicrographs). Grain density in controls (Fig. 4) approximated 11.7 ± 1.1 grains/oligodendrocyte (Fig. 4, vertical line column in graph below photomicrographs), contrasted with fewer grains in untreated EAE mice, about 7.4 ± 0.6 grains/oligodendrocyte (Fig. 4, dark column; $p < 0.01$ versus control). Treatment of EAE mice with the low PROG dose (20 mg) increased PLP mRNA grain density (Fig. 4) to 9.9 ± 0.4 grains/oligodendrocyte (Fig. 4 white column), which represented a 1.3-fold increase respect of untreated EAE (Fig. 4, $p < 0.01$). However, the stimulatory effect of 100 mg PROG (Fig. 4, horizontal line column) was minor and did not reach statistical significance. This was probably due to a mix response of oligodendrocytes in this group, since cells with high grain density coexisted with others showing low PLP mRNA expression (Fig. 4, EAE + PROG 100 mg).

3.4. PROG increases Na,K-ATPase mRNA in motoneurons of EAE mice

Although demyelination is a typical neuropathological finding of EAE, neuronal abnormalities frequently accompanied myelin deficiency [54,55]. Therefore, the effects of EAE with and without PROG treatment on the expression of the alpha 3 subunit mRNA for the NA,K-ATPase – an exclusive neuronal isoform – was investigated. In this experiment, neurons measuring >300 μm^2 localized in Lamina IX were considered to be α motoneurons based on anatomical location and size [56]. In control motoneurons, several grains

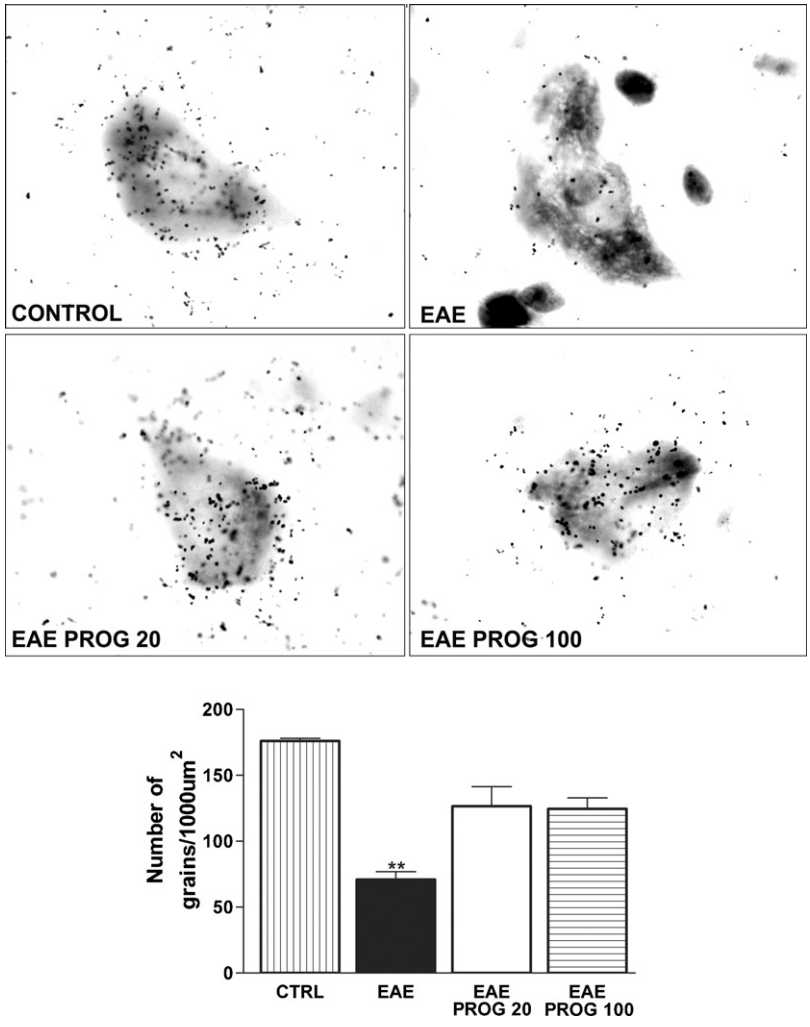

Fig. 5. *In situ* hybridization for neuronal Na,K-ATPase mRNA. The upper photomicrographs show that the EAE mouse contained fewer grains over the motoneuron cell body compared to a control, EAE + PROG 20 and EAE + PROG 100 mg-treated mice. Statistical analysis (lower graph) confirms that the number of grains per 1000 μm^2 was significantly lower in EAE mice than in the other three groups ($p < 0.01$). Data were gathered from ≈ 40 motoneurons contained in four spinal cord sections per animal ($n = 6$ mice per group).

representing oligonucleotide probe hybridized to the alpha 3 subunit of Na,K-ATPase mRNA were observed over the cell body (Fig. 5, top left photomicrograph). This pattern contrasted with the severe grain depletion in motoneurons of untreated EAE mice (Fig. 5, top right photomicrograph). PROG pellet implantation substantially recovered hybridization signal in EAE mice (Fig. 5, bottom left and right photomicrographs: PROG + 20 and PROG + 100 mg, respectively). Quantitative analysis of ISH data confirmed that EAE induced a 2.5-fold reduction of Na,K-ATPase mRNA when compared to control animals (Fig. 5, lower graph, vertical line column versus dark column; $p < 0.01$). In EAE mice treated with 20 mg or 100 mg PROG pellets (Fig. 5, white and horizontal line column), grain density doubled respect to untreated EAE animals ($p < 0.01$). Thus, PROG treatment successfully rescued alpha 3 Na,K-ATPase mRNA from the deleterious effect of EAE on ventral horn motor neurons.

4. Discussion

The present results demonstrated that administration of PROG has beneficial therapeutic effects in female mice with $MOG_{40–54}$-induced EAE. PROG attenuated disease severity, delayed disease onset and improved spinal cord neuropathology.

All $MOG_{40–54}$ immunized mice were clinically afflicted with EAE, and the first signs of the disease appeared at day 9–10 following immunization. Mice in the EAE steroid-free group reached grade 4 of the disease, characterized by complete loss of tail tonicity, limb paralysis and pronounced motor impairment on day 16 after immunization. In contrast, EAE signs were slightly although significantly retarded by 1–2 days in animals treated with the low and high PROG doses, respectively. These groups of PROG-treated EAE mice still showed a loss of tail tonicity but paralysis was milder, motor impairment less pronounced and they

did not go beyond grade 3 on average. Disease index, an appropriate parameter determining disease progression, was markedly decreased by PROG pellet implantation in comparison to steroid-naïve EAE mice. Therefore, although PROG partially prevented development of the disease, results were encouraging and may be of pharmacological interest for the therapeutic intervention of EAE.

At the spinal cord level, EAE mice showed the expected inflammatory cell infiltration, partly composed of cells belonging to a macrophage lineage, whereas infiltration was reduced by PROG treatment. Existing data suggest that similarly to the effects of pregnancy, PROG changes a Th1 pro-inflammatory response to a Th2 anti-inflammatory response and decreases inflammatory mediators [16–21]. Therefore, it is possible that PROG displays similar effects in EAE mice. In our case, implantation of 20 and 100 mg PROG pellets significantly dampened infiltration of inflammatory cells into the spinal cord, supporting its modulatory role upon the immune reaction sparkled by EAE. The levels of serum progestins in animals implanted with the 20 mg pellet were in the range of pregnant mice at days 19–20 of pregnancy ($\approx$ 40 ng/ml, unpublished data). With the 100 mg pellet, levels were pharmacological for the mouse. In Wobbler mice receiving 20 mg PROG pellets, levels of progestins were similar to those of 20 mg-implanted EAE mice. These Wobblers show improvement of clinical signs and motoneuron pathology [8], suggesting the $\approx$ 40 ng/ml levels are sufficient for neuroprotection and to produce beneficial effects in spinal cord neurodegeneration.

EAE is considered a primary demyelinating disease caused by oligodendrocyte degeneration [57] and a good model to test factors increasing myelination. In our mice patchy demyelinating lesions, characteristics of EAE, were confirmed by the reduced LFB staining and focalized loss of MBP and PLP immunoreactivity. Reduced levels of PLP mRNA also suggested an effect on gene expression. The focal areas of demyelination were more frequently observed in the ventral and dorsal funiculus. This abnormality lies behind the clinical signs such as motor impairment and paralysis, because in rodents the corticospinal tract runs in the dorsal funiculus, whereas ventral roots are composed by motor axons derived from ventral horn motoneurons [56]. The loss of myelin was prevented by PROG. In this case, myelin recovery in the dorsal and ventral funiculus of the spinal cord was probably behind the improvement of clinical scores of EAE mice. Regarding PLP mRNA, it is interesting that the low but not the high dose of PROG increased its expression. As already pointed out, this was due to differential effects of the hormone on oligodendrocytes. It has been reported that high PROG doses are less effective than lower doses in cases of brain traumatic injury [21]. In the case of EAE mice, further experiments are needed to re analyze this issue, such as finding a threshold dose of PROG that could prevent or attenuate signs of the induced disease.

Although the anti-inflammatory effect of PROG may be an important determinant of myelin preservation in EAE, the effect on myelin proteins and PLP mRNA suggest that PROG may directly regulate myelin in oligodendrocytes and/or stimulate the proliferation and differentiation of oligodendrocyte precursors, as published for other experimental paradigms [9–14,58]. The detection of PROG receptors (PR) in oligodendroglia supports a direct action on myelin-producing cells and their progenitors [15,59]. Based on these evidences, it is possible that PROG exerts a dual role, anti-inflammatory and promyelinating, in the spinal cord of EAE mice.

The present results confirmed neuronal involvement in EAE mice, due to the fact that the Na,K-ATPase mRNA was reduced. The enzyme, which is hormone sensitive in the spinal cord [4], regulates excitability, neurotransmission and nutrient uptake, besides its ion transport properties [60,61] and its deficit is a leading cause of neurodegeneration [62]. Thus, a decrease of the Na,K-ATPase mRNA, as occurred in untreated EAE mice, could lead to neuropathology and motor impairment. Up-regulation of Na,K-ATPase mRNA by PROG, as reported early in spinal cord injury and Wobbler neuron degeneration [4,7] may afford neuroprotection.

In conclusion, PROG attenuated neuropathology and clinical signs of EAE. Downregulation of the immune response is likely involved, although remyelination and neuroprotection may be additional mechanisms. Future experiments will investigate if PROG enhancement of myelin proteins in EAE is accompanied by proliferation/differentiation of oligodendrocyte precursors. These cells are responsible for remyelination, but this event is incomplete and poorly sustained in multiple sclerosis [63–65]. Since PROG modulates the oligodendrocyte lineage in culture and increases precursor proliferation after spinal cord damage [13,14] it would be important to investigate this possibility in EAE mice.

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