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Progesterone treatment modulates mRNA OF neurosteroidogenic enzymes in a murine model of multiple sclerosis

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ABSTRACT

Previous studies of experimental autoimmune encephalomyelitis (EAE) have shown that progesterone decreases inflammatory cell infiltration and proinflammatory factors, increases myelination and attenuates clinical grade of EAE mice. To elucidate potential mediators of these effects, we analyzed the mRNA expression of neurosteroidogenic enzymes in the spinal cord, in view of the protective role of steroids in EAE. We also analyzed mitochondrial morphology and dynamics (fusion and fission proteins), considering the role of mitochondria in neurosteroidogenesis. EAE was induced in C57Bl6 mice using MOG_{40-54} and killed on day 16 after induction. Using qPCR, we found in steroid-untreated EAE mice decreased mRNAs for the steroidogenic acute regulatory protein (Star), voltage-dependent anion channel (VDAC), P450scc (cholesterol side-chain cleavage), 5α-reductase, 3α-hydroxysteroid dehydrogenase (3α-HSD) and aromatase, whereas levels of 3β-hydroxysteroid dehydrogenase (3β-HSD) showed a large intra-group variance. We also found increased mRNA expression of 18 Kd translocator protein (TSPO), which likely resulted from the reactive microgliosis in this model. EAE mice also showed pathological mitochondrial morphology and reduced expression of fission and fusion protein mRNAs. Most importantly, pretreatment with progesterone a week before EAE induction increased Star,VDAC, P450scc, 5α-reductase type I, 3α-HSD and aromatase mRNAs and did not modify 3β-HSD. TSPO mRNA was decreased, consequent with the inhibition of microgliosis. Mitochondrial morphology was improved and fission/fusion protein mRNAs were enhanced by progesterone treatment. Furthermore, progesterone protective effects on mitochondrial and endoplasmic reticulum may allow the recovery of neurosteroidogenesis. In this way, endogenously synthesized neurosteroids may reinforce the beneficial effects of exogenous progesterone previously shown in MS mice.

1. Introduction

Multiple sclerosis (MS) is a neurological disorder that affects about 2.5 million people worldwide, according to the World Health Organization. MS strikes the spinal cord, brain and optic nerves with a female to male incidence of 2:1 [1]. In about 80% of the patients, it shows a relapsing-remitting course [2]. Based on this outcome, MS has been considered of autoimmune origin, due to

Abbreviations: CC1, anti-adenomatours polyposis coli clone CC1; CD11b, cluster of differentiation molecule 11B; CFA, complete Freund adjuvant; DHP, dihydroprogesterone; EAE, experimental autoimmune encephalomyelitis; Fis1, mitochondrial fission 1; GABA, gamma-aminobutyric acid; 3α-HSD, 3 alpha-hydroxysteroid-dehydrogenase; 3β-HSD, 3 beta-hydroxysteroid-dehydrogenase; PS, lipopolysaccharide; MBP, myelin basic protein; Mfn2, mitofusin 2; MOG, myelin oligodendrocyte protein; MS, multiple sclerosis; Nkx2.2, NK2 Homeobox 2; NMDA, *N*-methyl-D-aspartate; Olig1, oligodendrocyte Transcription Factor 1; PLP, proteolipid protein; PR, progesterone receptor; PRMC1, progesterone receptor membrane component 1; PROG, progesterone; THP, tetrahydroprogesterone; TLR4, toll-like receptor 4; TNFa, tumor necrosis factor alpha; TNFR1, tumor necrosis receptor type 1; TSPO, mitochondrial translocator protein 18 Kd; Star, steroidogenic acute regulatory protein; VDAC, voltage-dependent anion channel.

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the infiltration of peripheral immune cells into the central nervous system (CNS). Inflammatory infiltrates and activated microglial cells produce proinflammatory factors causing the loss of oligodendrocytes, disruption of the myelin sheath and impairment of axonal conductance [3,4]. In addition to the autoimmune origin, a neurodegenerative hypothesis for MS has also been advanced, emphasizing primary defects of oligodendrocytes and neurons [5,6]. Regardless of its origin, a role for steroid hormones in MS is suggested based on the high female to male ratio and because relapses decline during the last trimester of pregnancy, when estrogens and progesterone levels are high, and resume when steroids decay after delivery [7–10]. This inverse relationship has encouraged several clinical and experimental studies to elucidate the role of gender and sex steroids in MS.

Measurements of circulating and brain steroid levels reinforced the participation of steroids in MS pathophysiology. Noorbakhsh et al. [18] have shown reduced levels of the progesterone derivative allopregnanolone (ALLO) and low levels of the enzymes converting progesterone into dihydroprogesterone (DHP) and ALLO in the brain from MS patients. In the same vein, Caruso et al. [19] have reported changes of progesterone and testosterone metabolites in cerebrospinal fluid and plasma of relapsing-remitting MS patients. In dark Agouti rats with induced experimental autoimmune encephalomyelitis (EAE), Giatti et al. [11] have shown a dimorphic profile in male and female rats regarding progesterone and testosterone metabolites. After progesterone treatment, these rats showed increased progesterone, DHP and isopregnanolone levels in the spinal cord [11]. Thus, profound changes in the levels of steroids secreted by endocrine glands as well as in brain-synthesized steroids (neurosteroids) occur in MS patients and rodent models of the disease.

Our group and other laboratories have reported that progesterone and synthetic progestins show beneficial effects in EAE and other forms of induced demyelination [12–15]. Regarding the mechanism(s) responsible for these effects, it should be recalled that progesterone is a multifaceted hormone that binds to different receptors and interacts with a number of intracellular pathways [16,17]. In the case of MS patients and preclinical models of the disease, the reduced levels of progesterone metabolites and steroidogenic enzymes point to faulty neurosteroidogenesis; the latter may aggravate neuropathology [11,18,19]. From the pioneering work of Baulieu [20], it is widely acknowledged that the CNS synthesizes its own steroids, named “neurosteroids”. Enzymes of steroid synthesis are expressed by neurons, astrocytes, oligodendrocytes and microglia, suggesting that these cell types work in concert [21]. The locally-produced neurosteroids are not secreted to the periphery but act in a paracrine/autocrine way to regulate gene expression, modulate GABA(A), NMDA and sigma receptors, increase myelin synthesis, exert neuroprotective and anti-inflammatory effects, and control the growth of axons, dendrites and spines [22–26].

In the present study, we hypothesize that part of progesterone neuroprotection in EAE may be due to up-regulation of neurosteroidogenesis. Employing qPCR technology, we quantified changes in mRNA expression of the mitochondrial transduceosome, including the steroidogenic acute regulatory protein (Star), voltage-dependent anion channel (VDAC) and the 18-Kdal translocator protein (TSPO). The mRNA of the mitochondrial P450scc (cyp11a1), which metabolizes cholesterol to pregnenolone, was also measured. Changes in the mRNA for the transduceosome complex and P450scc may be reflected by their respective mitochondrial proteins, because mitochondria found in axons and neuronal bodies are largely abnormal in both MS and the EAE model [27,28]. Therefore, the present study also assessed the effects of EAE and progesterone treatment on mitochondrial ultrastructure and mitochondrial dynamics involving fusion and fission proteins. Disruption of mitochondrial dynamics occurs in Alzheimer and Parkinson diseases, neurodegeneration and EAE mice [29–31].

Our search for progesterone effects on neurosteroidogenesis in EAE also included the mRNAs of the microsomal enzymes 3β-hydroxysteroid dehydrogenase type I (3β-HSD, converting pregnenolone to progesterone), 5α-reductase type I (converting progesterone to DHP), 3α-hydroxysteroid dehydrogenase type I (3α-HSD, converting DHP to allopregnanolone) and aromatase (converting androgens to estrogens). Additionally, the enzymes 3β-HSD, 3α-HSD and 5α-reductase are also responsible for the formation of androgens. Androgen levels are modified in MS patients and EAE models [11,19], and together with progestins and estrogens, may play a neuroprotective role for EAE and MS outcome [12–15,32]. The present work, however, was mainly focused on progestins. In this sense, the clinical and neurochemical effectiveness of the progesterone treatment protocol used in the present work has been validated in EAE mice studied in parallel to the present investigation. Our prior publications demostrate that progesterone attenuates clinical grade, decreases proinflammatory factors, microgliosis and astrogliosis, and increases myelin formation in EAE mice [33–35].

2. Materials and methods

2.1. Experimental animals

Nine to 11 week-old female C57BL/6 mice were purchased from the Faculty of Veterinary Medicine (La Plata, Argentina). Mice were housed with food and water ad libitum, and maintained under constant temperature on a 12 h light/dark cycle. To induce EAE, mice received by sc injection on each flank 200 μg MOG_{40-54} peptide (Peptides International, LA, USA) [33,34,36] emulsified in complete Freund’s adjuvant (CFA) containing 0.6 mg *Mycobacterium tuberculosis* (Instituto Malbran, Argentina). The animals also received i.p. 400 ng injections of *Pertussis* toxin (Sigma) at the time of immunization and another boost on the next day. Some animals received CFA and *Pertussis* toxin without MOG but none of them developed signs of EAE. EAE mice remained untreated or received subcutaneously (sc) a single 100 mg progesterone pellet (Sigma Chem. Co, St.Louis, MO) one week before disease induction, following a previously used protocol [33,34]. One cohort of EAE mice was used to asses progesterone effects on clinical grade, spinal cord inflammation and microglial reaction [35], whereas a second cohort was used for expression analysis of mitochondrial proteins involved in cholesterol transport and steroidogenic enzymes. In progesterone-free animals, EAE developed approximately on day 10 and they were sacrificed on day 16 when the disease was still in an acute phase [33].

Animal procedures followed the Guide for the Care and Use of Laboratory Animals (NIH Guide). Our institution was approved by the Office of Laboratory Animal Welfare (OLAW, NIH, USA) through the Assurance Certificate # A5072-01 effective until March 2019. Experimental animal protocols were also approved by the Institute's Animal Care and Use Committee (IACUC).

2.2. Real time PCR of neurosteroidogenic enzymes and fusion/fission proteins in the spinal cords

For real time PCR, mice were deeply anesthetized with a mixture of xylazine (6 mg/kg) and ketamine (75 mg/kg) and killed by decapitation. A 0.5 cm segment of the cervical spinal cord was removed and homogenized with a Polytron homogenizer. Total RNA was then extracted using Trizol reagent (Life Technologies, Invitrogen). The concentration and purity of total RNA was determined by measuring the optical density at 260 and

280 nm. All samples were precipitated with ethanol and then dissolved in distilled water at a concentration of 1 μg/μL. Total RNA was subjected to Dnase 1 (InvitroGen) treatment (2U for 10 min at room temperature) to remove residual contaminating genomic DNA. cDNA templates for PCR amplification were synthesized from 1 ng of total RNA using a MMLV High Performance reverse transcriptase (Epicentre, USA) for 60 min at 37° C in the presence of random hexamer primers. Table 1 shows the forward and reverse primers employed for Star, TSPO, VDAC, P450scc, 3β-HSD, 3α-HSD, 5α-reductase typeI, aromatase, mitofusin 2 (Mfn2) and mitochondrial fission 1 protein (Fis1). Cyclofilin B was used as a housekeeping gene based on the similarity of mRNA expression across all samples templates. The relative gene expression for different mRNAs was determined using the ABI PRISM 7500 sequence Detection System (Applied Biosystems, Foster City, CA). Relative gene expression data were calculated using the $2^{-\Delta\Delta ct.}$ method [37], and it was determined as fold induction with respect to its respective control. Working conditions for amplification have been already described [35]. At least two replicates were analyzed per gene target for each sample. *We* used 6–7 animals per experimental group, composed of controls, EAE and EAE plus progesterone-treated mice.

2.3. Electron microscopy studies

For electron microscopy, mice were anesthetized and perfused transcardially with 4% paraformaldehyde containing 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.4. Spinal cords were removed and blocks of tissue were obtained by cutting transverse sections of 2–3 mm maximum length. Blocks were immersed for 2 h in the same fixative. After overnight washing in 0.1 M sodium phosphate buffer, tissue blocks containing the ventral horns were postfixed in 1% OsO_4 in 0.1 M phosphate buffer pH 7.4 for 1 h and stained with1% uranyl acetate. Afterwards, tissue blocks were dehydrated and flat-embedded in Durcupan (Fluka Chem. AG, Sweden). Ultrathin sections (60–70 nm) were obtained with a Reichert ultramicrotome (Vienna, Austria). Sections were stained with lead citrate, examined at 85,000× magnification and photographed using a Zeiss 109 electron microscope facility at the Faculty of Medicine, University of Buenos Aires.

2.4. Statistical analysis

Results were expressed as the mean ± SEM. Comparison between groups was carried out by ANOVA followed by the post-hoc Newman-Keuls multiple comparison tests. Statistical analysis was performed with PRISM 4 (GraphPad Prism software, San Diego, CA, USA). The level of significance was set at $p < 0.05$.

3. Results

3.1. Effects of EAE and progesterone treatment on the expression of mitochondrial neurosteroidogenic enzymes

We first studied the mRNA of the mitochondrial transduceosome complex in charge of transporting extra-mitochondrial cholesterol to the inner mitochondrial membrane. Fig. 1 shows the expression profile of Star, TSPO and VDAC mRNAs in the control, EAE and progesterone-receiving EAE mice. EAE caused marked decreases in their mRNA levels, with the exception of TSPO, whereas progesterone treatment resulted in the opposite effect. Thus, Star mRNA was reduced by 40% in EAE ($p < 0.01$ vs. control) and it was significantly increased in progesterone-receiving EAE mice ($p < 0.01$ vs. untreated EAE). Up-regulation in the steroid-treated group made Star mRNA levels similar to those of the control group (p: NS). VDAC mRNA was also markedly depleted by 80% in the spinal cord of EAE mice ($p < 0.001$ vs. control), returning to control levels in the progesterone-treated EAE group ($p < 0.001$ vs untreated EAE; p:NS vs. control). In the same trend of Star and VDAC, the P450scc mRNA showed a pronounced 84% decrease in EAE mice ($p < 0.05$ vs control), with complete recovery when EAE mice received progesterone (EAE + progesterone vs. untreated EAE: $p < 0.01$). No differences were found between the last group and the control mice (p:NS).

In contrast to Star, VDAC and P450scc mRNA, TSPO mRNA showed a 6-fold significant increase in untreated EAE mice compared to the control group (Fig. 1) ($p < 0.001$). Progesterone pre-treatment of EAE mice significantly reduced TSPO mRNA ($p < 0.05$). The result obtained with TSPO was not unexpected, since microglia are the predominant source of TSPO [21,38–41]. Microglial cells are up-regulated in EAE-induced neuroinflammation and decrease after treatment with progesterone or progestins [35,42], indicating that variation in TSPO expression followed the response of microglia number and activation.

3.2. Effects of EAE and progesterone treatment on the expression of microsomal neurosteroidogenic enzymes

Since completion of neurosteroidogenesis requires the combined action of mitochondrial and microsomal-located enzymes, we performed quantitative analysis of the mRNAs of the microsomal enzymes 3β-HSD1, 5α-reductase, 3α-HSD and aromatase. Levels of mRNAs were assessed in control, EAE and progesterone-treated EAE mice (Fig. 2). A marked variability in the expression of 3β-HSD1 mRNA in the EAE group precluded drawing valid conclusions regarding group differences. Instead, we found a marked depletion of 5α-reductase ($p < 0.001$), 3α-HSD ($p < 0.01$)

Table 1
Sequence of primers for neurosteroidogenic proteins and enzymes and fission (Fis1) and fusion (MnF2) proteins used for real-time PCR.

Gene	Accesion Number	Primer sense 5′ – 3′	Primer antisense 5′ – 3′
Cyclophilin b	NM_001025612	GTGGCAAGATCGAAGTGGAGAAAC	TAAAAATCAGGCCTGTGGAATGTG
StAR	NM_011485	GAGCTCTCTGCTTGGTTCTAA	TTGAGTATGCCCAAGGCCTT
TSPO	NM_009775	TGCAGAAACCCTCTTGGCATC	TGAAACCTCCCAGCTCTTTCC
VDAC	NM_011694	CTCCCACATACGCCGATCTT	GCCGTAGCCCTTGGTGAAG
P450scc	NM_019779	CCTATTCCGCTTTTCCTTTGAGTCC	CGCTCCCCAAATATAACACTGCTG
3β-HSD1	NM_008293	TCTGAAAGGTACCCAGAACCTATTGG	TTGCTTGAACACAGGCCTCCA
5α-Reductase	NM_175283	TGTTTCCTGACAGGCTTTGCCC	CCATGCCCACTAACCACAGGG
3α-HSD1	NM_134072	CACATTGGGAAGTTCACGAGACA	AAGCCAACTGGAATTCAAAAACCT
Aromatase	NM_007810.3	CGGGCTACGTGGATGTGTT	GAGCTTGCCAGGCGTTAAAG
Fis 1	NM_001163243.1	CTACAGGGGTGCAGGAGAAA	AGATGGACTGGTAGGCATGG
Mfn 2	NM_001285920.1	CATCAGTTACACCGGCTCTAACT	GAGCCTCGACTTTCTTGTTCA

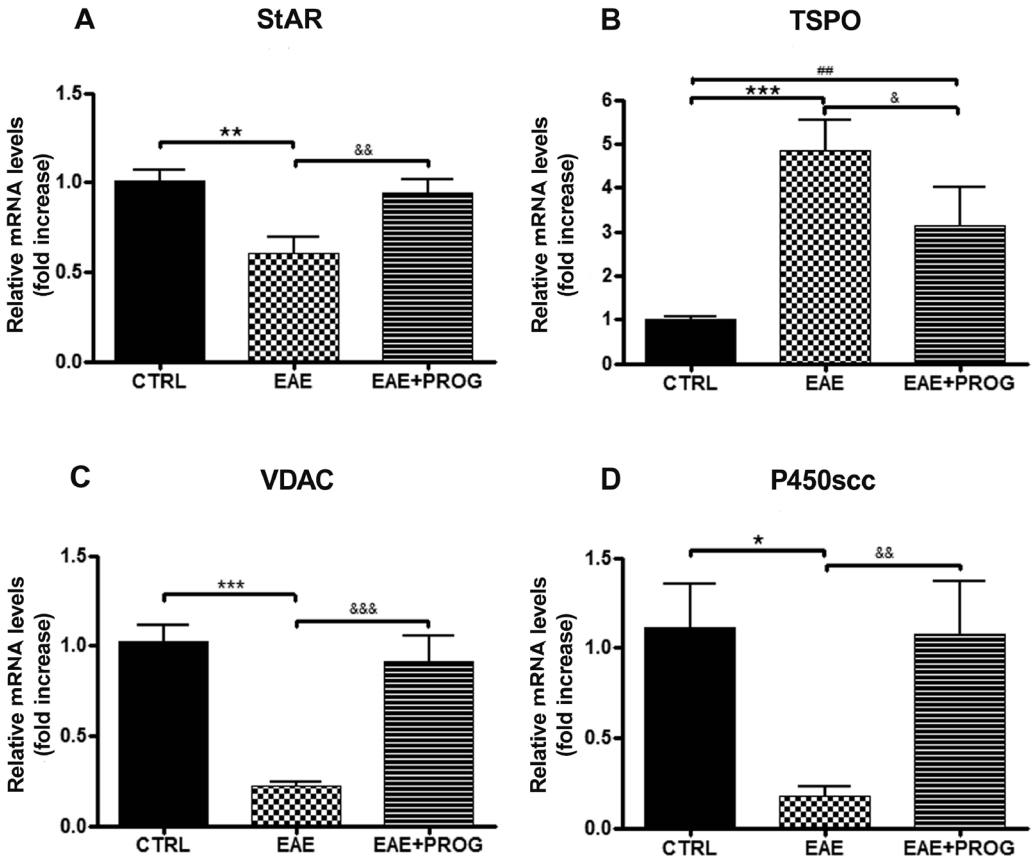

Fig. 1. mRNA levels (mean +/− SEM) of mitochondrial proteins forming the transduceosome and mitochondrial P450scc in the spinal cord from control, EAE and EAE mice receiving progesterone (PROG) pretreatment. Results are plotted as relative changes (mean ± SEM) with control mRNAs taken as 1.0. A: Levels of the steroidogenic acute regulatory protein (Star) were decreased in EAE mice (** $p < 0.01$ vs control) and increased in the EAE + PROG group (&& p0.01 vs EAE). B: Translocator protein 18 K (TSPO) mRNA was increased in EAE (*** $p < 0.001$) and decreased in EAE + PROG mice ($^{\&}$ $p < 0.05$). The mRNA of the EAE + PROG group remained higher than control ($^{\#\#}$ $p < 0.01$). C: Levels of the voltage-dependent anion channel (VDAC) were decreased in EAE (***$p < 0.001$) and increased in EAE + PROG mice ($^{\&\&\&}p < 0.001$). D: mRNA of the cholesterol-side chain cleavage enzyme (P450scc) was decreased in EAE (*$p < 0.05$) and increased in the EAE + PROG mice ($^{\&\&}p < 0.01$). Data obtained were analyzed by ANOVA followed by the Newman-Keuls test (n = 6–7animals per group).

and aromatase mRNAs ($p < 0.01$) in EAE mice vs. control mice. Furthermore, and in consonance with changes in Star, VDAC and P450scc, progesterone pre-treatment significantly increased 5α-reductase ($p < 0.001$), 3α-HSD ($p < 0.05$) and aromatase ($p < 0.01$) mRNAs compared to steroid-untreated EAE mice (Fig. 2). Thus, after progesterone treatment, EAE mice no longer differ from controls (Fig. 2, EAE+ progesterone vs. control: NS).

3.3. Electron microscopy and qPCR of fusion/fission proteins of mitochondria in control, EAE and progesterone-pretreated EAE mice

Since the transduceosome is a mitochondria-located complex, and aberrant mitochondria have been reported in neurons from MS and EAE [27,28], we examined whether progesterone preserved the morphology of this organelle in EAE mice. EM observations were performed on motoneurons from the ventral horn, focusing on distal, axonal mitochondria and those proximal to the neuronal body. In Fig. 3, picture A represents intact mitochondria inside a well myelinated axon from a control mouse, which appears in the process of division, whereas image B shows control mitochondria within the neuronal body displaying well-preserved cristae. In contrast, an EAE mouse (C) showed intensely vacuolated mitochondria inside an axon with highly disrupted myelin sheath. In the cell body (D), the EAE mouse also showed a membrane-disrupted, vacuolated mitochondria without visible cristae. Image E, taken from a progesterone-pretreated EAE mouse (E) shows two small mitochondria with conserved ultrastructure inside an axon with better preserved myelin sheath. In the photomicrograph from a progesterone-treated mouse (F) a mitochondrion with a better preserved ultrastructure was seen in the neuronal body. Therefore, mitochondria located at proximal or axonal sites in the neuron showed a more preserved ultrastructure after progesterone treatment, compared to steroid-naïve EAE mice.

Fig. 4 shows changes of the mRNA for Fis 1 and Mfn2, key molecules involved in mitochondrial dynamics. Fis1 mRNA was reduced by 1.6-fold in the spinal cord of EAE mice vs. control mice ($p < 0.001$), whereas progesterone treatment increased the expression of this molecule by 25% in EAE mice ($p < 0.05$ vs untreated EAE). Mfn2 mRNA was reduced by more than 3-fold in EAE spinal cord, and its increase after progesterone treatment was highly significant ($p < 0.001$ vs untreated EAE). Therefore, changes of the expression of molecules associated with mitochondrial dynamics are consistent with the alterations of mitochondrial morphology observed by electron microscopy.

4. Discussion

The present investigation demonstrates that the decreased expression of mitochondrial proteins and enzymes involved in neurosteroidogenesis in the spinal cord of EAE mice were prevented by progesterone pretreatment. Our study began with measurement of the mRNAs of the multimeric protein complex

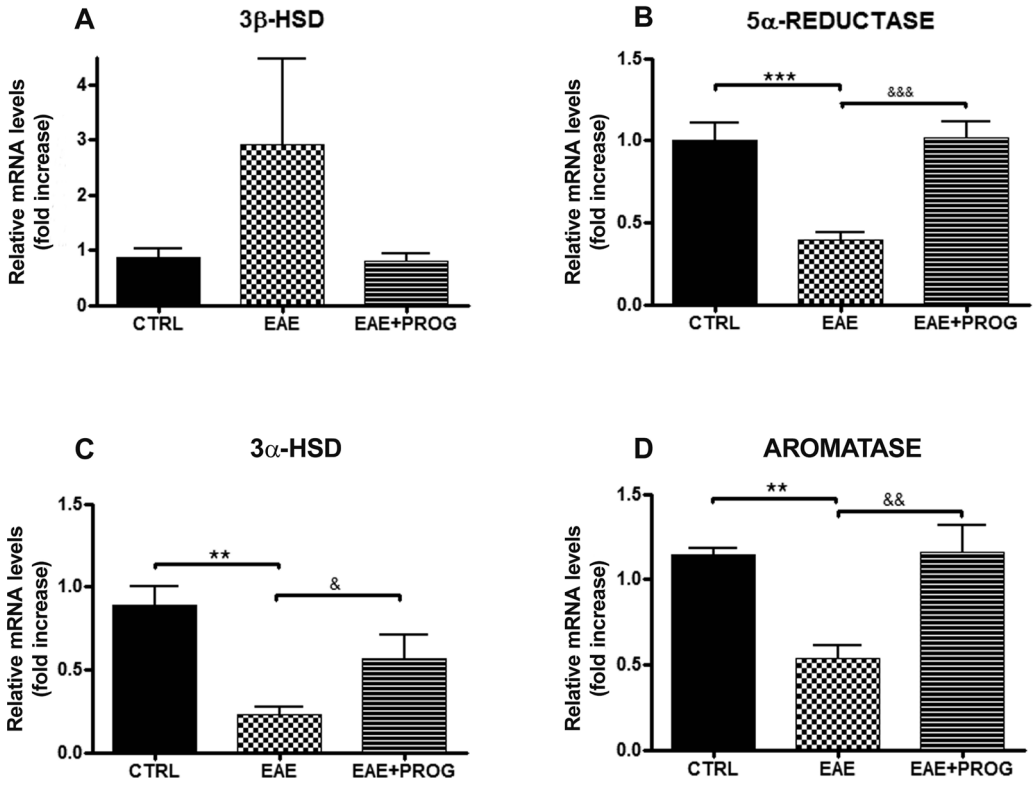

Fig. 2. mRNA levels (mean +/− SEM) of microsomal neurosteroidogenic enzymes in the spinal cord from the groups depicted in the legend of Fig. 1. A: Variations of the mRNA of 3β-hydroxysteroid dehydrogense (3β-HSD) did not significantly differ between control, EAE and EAE + PROG groups. B. Levels of mRNA for 5α-reductase were decreased in EAE (***$p < 0.001$) and increased in EAE + PROG-treated mice ($^{\&\&\&}p < 0.001$). The last group was not different from controls (p:NS). C: mRNA of 3α-hydroxysteroid dehydrogenase (3α-HSD) was decreased in EAE (**$p < 0.01$) and increased in the EAE + PROG group ($^{\&}p < 0.05$). The low mean of the last group was not significantly different from controls (p:NS). D: Aromatase mRNA was lower in EAE (**$p < 0.01$ vs. control) and increased in the EAE + PROG-treated mice ($^{\&\&}p < 0.01$). The last group and control measured similarly. Data obtained by ANOVA followed by Newman-Keuls tests (n = 6–7 animals per group).

known as the transduceosome [41]. Results show that two of these components (i.e. Star and VDAC) were substantially reduced in EAE mice, indicating that EAE may impair the transport of cholesterol from cytoplasmic stores into the mitochondria, where the first step in steroid synthesis takes place. In addition, reduced mRNA for P450scc in the EAE group suggested a reduced capacity of converting cholesterol to pregnenolone, the first step in the steroidogenic pathway. Furthermore, EAE mice also showed decreased mRNAs of microsomal enzymes. Thus, low expression of 5α-reductase and 3α-HSD mRNA suggest that progesterone metabolism into its reduced derivatives DHP and ALLO may also be impaired. In this context, reduced levels of DHP have been reported in EAE rats [11] and low levels of ALLO have been described in the brain of MS patients and EAE mice [18]. Although our studies did not localize the cellular origin of the enzymes required for DHP and THP production, Patte-Mensah et al. [43] first demonstrated that oligodendrocytes and neurons possess the enzymatic machinery for synthesizing these potent neurosteroids.

Reduced levels of transduceosome components indicate that EAE may damage the cell types and organelles expressing these molecules. For example, low expression levels of Star, VDAC and P450scc strongly suggest that their respective functions may be decreased. Star is found in mitochondria from neurons and astrocytes according to Sierra et al. [44] and King and Stocco [45]. Using double immunofluorescence with Star and CC1 antibodies, we have also observed localization of Star in oligodendrocytes (Garay et al., unpublished data). In the case of VDAC, it is found predominantly in neuronal mitochondria [41], whereas P450scc is a mitochondrial-based enzyme found in neurons, astrocytes and oligodendrocytes [46,47]. In contrast to Star and VDAC, TSPO mRNA was significantly up-regulated in EAE mice. TSPO is found in mitochondria from microglia and astrocytes, both of which become highly reactive in EAE mice [35]. It is known that CNS injury, lipopolysaccharide (LPS) treatment and EAE are potent inducers of TSPO, a molecule associated with highly reactive microglia [21,38–41]. These observations explain the dissociation in the expression of transduceosome mRNA components in EAE neuroinflammation.

Furthermore, EAE mice also showed decreased mRNAs of microsomal enzymes. Thus, low expression of 5α-reductase and 3α-HSD mRNA would imply that progesterone metabolism into its derivatives DHP and ALLO was also reduced. In this context, reduced levels of DHP have been reported in EAE rats [11] and low levels of ALLO have been described in the brain of MS patients and EAE mice [18], in consonance with our studies. 5 α-reductase is found in astrocytes and oligodendrocyte precursors [48,49]. The product of this enzyme, DHP, is a ligand of the PR, and acting through this receptor mediates neuroprotective actions of progesterone in Dark Agouti rats with EAE according to Giatti et al. [11]. 3α-HSD reduces DHP into ALLO, a positive modulator of GABA(A) receptors, which may counterbalance excitotoxic neurotransmission arising during CNS insults. Indeed, treatment with ALLO or increasing GABAergic activity with diazepam ameliorates EAE in mice and rats, respectively [18,50], supporting a neuroprotective role of THP in EAE.

We also found reduced aromatase mRNA expression in EAE, which may reflect impaired conversion of androgens to estrogens, depriving EAE mice of neuroprotective estrogens. Aromatase is

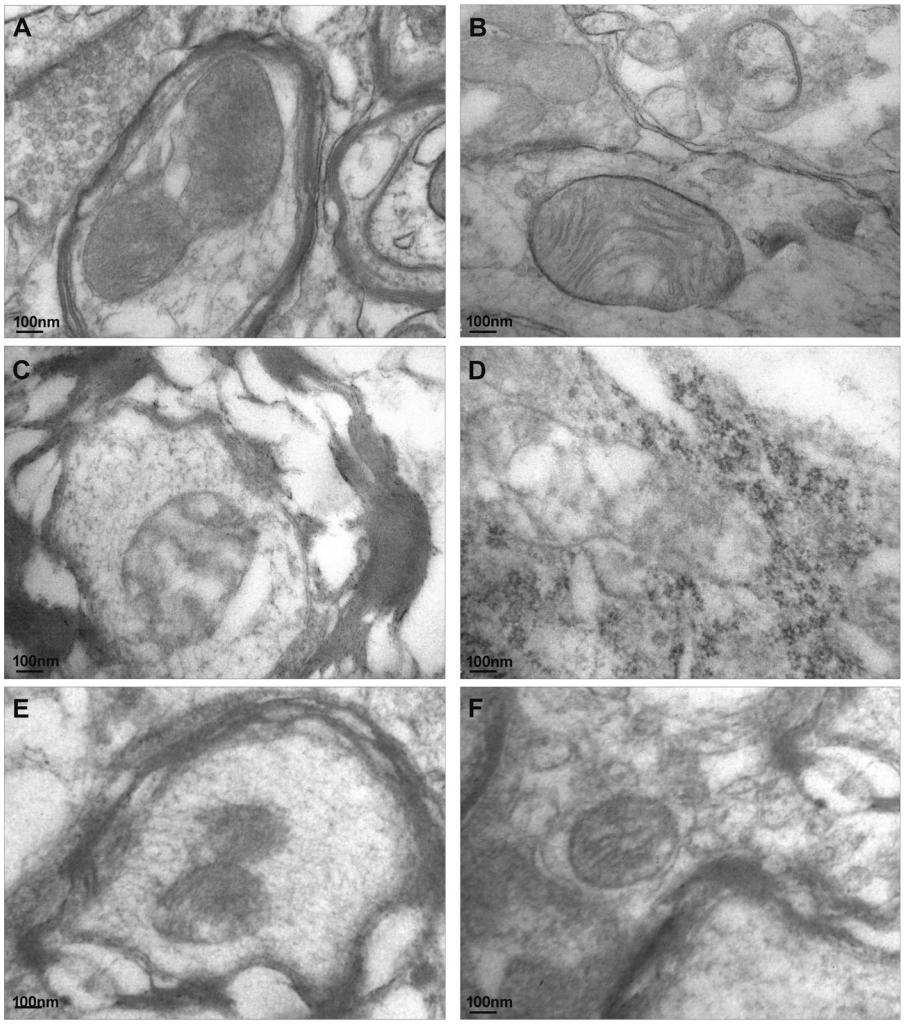

Fig. 3. Mitochondrial ultrastructure in control, EAE and EAE + PROG groups. Electron microscope observations were focused on distal, axonal mitochondria (A, C. E) and proximal mitochondria (B, D, F). A and B: photomicrographs of control mitochondria inside a myelinated axon (A) and in the motoneuron cytoplasm (B) showed intact mitochondria with well-preserved cristae. Mitochondrion in A is probably in the process of fission. C: image of a damaged vacuolated mitochondria inside an axon with disrupted myelin sheath. D: a proximal, intensely vacuolated organelle without cristae in an EAE mouse. E and F: photomicrographs of distal mitochondria with visible cristae inside a myelinated axon (E) and a proximal mitochondria with well preserved cristae (F) from an EAE + PROG mouse. Vacuoles were absent from mitochondria shown in E and F. Magnification: 85,000×.

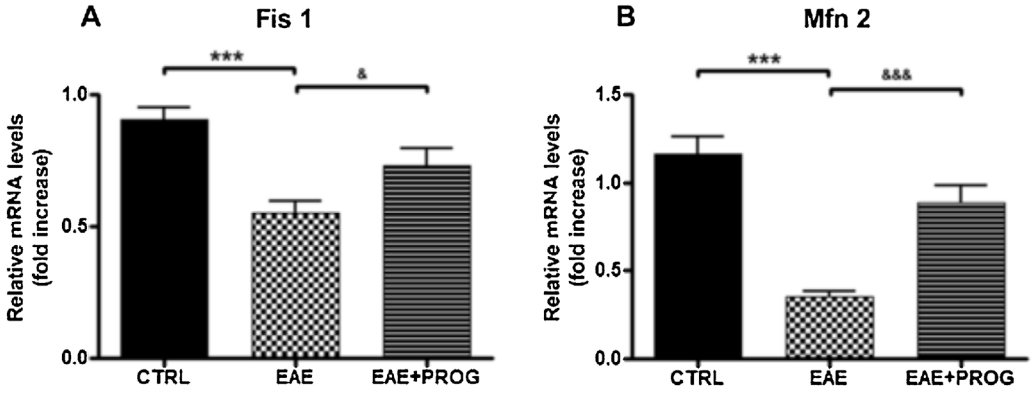

Fig. 4. mRNA levels (mean +/− SEM) of the mitochondrial fission 1 protein (Fis1) and mitofusin2 protein (Mfn2) from the animal groups depicted in the legend of Fig. 1. A: mRNA levels for Fis 1 were decreased in EAE (*** $p < 0.001$ vs. controls) and increased in the EAE + PROG group ($^{\&}p < 0.05$ vs EAE). B: Mfn2 was also decreased in EAE (*** $p < 0.001$ vs controls) and increased in the EAE + PROG group ($^{\&\&\&}p < 0.001$ vs EAE). Data obtained were analyzed by ANOVA followed by the Newman-Keuls test (n = 6 animals per group).

normally found in neurons but can be expressed by astrocytes after CNS injury or in Alzheimer's disease [51,52]. In connection to our investigation, it is known that estrogens are potent immunomodulatory factors for MS preclinical models, either alone or combined with progesterone [32]. In summary, reduced expression of 5α-reductase, 3α-HSD and aromatase in EAE mice indicates that steroid production in the endoplasmic reticulum may be compromised in EAE. The expression of 3β-HSD in untreated EAE mice, a molecule mainly found in microsomes [47,53] did not follow the decline of the other microsomal neurosteroidogenic enzymes and showed great variability. Similarly, Noorbakhsh et al. [18] have reported unchanged expression of 3β-HSD in EAE mice. The behaviour of 3β-HSD in EAE seems different from brain injury, which produces its down-regulation [53], but similar to spinal cord injury, in which no change was demonstrated [54].

It is likely that decreased expression of the mRNA of neurosteroidogenic enzymes, as shown here and elsewhere plus reduced neurosteroid levels [11,18], contributed to the worsening of EAE. In fact, in a cohort of EAE mice studied in parallel with those employed here, we have shown high clinical scores, demyelination and increased expression of proinflammatory factors and oxidant enzymes in the spinal cord, supporting the idea that reduced neurosteroidogenesis may worsen neuropathology [35]. It is possible, then, that the abnormal mitochondrial morphology and changes of mitochondrial dynamics in EAE mice may be partly due to the lack of neuroprotective steroids acting upon this organelle. In fact, EM observations demonstrated a disorganized mitochondrial ultrastructure at the cell body and axons of EAE, in contrast to a better organized mitochondrial profile in a progesterone-treated-EAE mouse. It is recognized that this qualitative appraisal will require a count of affected mitochondria in each model to ascertain this point. However, quantitative measurements by qPCR showed changes of fusion and fission mitochondrial proteins in EAE, as shown by reduced expression of Fis1 and Mfn2 mRNAs. Alterations of fusion and fission are associated with neurodegenerative diseases [30,31], and these have already been reported in the spinal cord of EAE mice [29]. Abnormalities of the mitochondrial function may impair the entry of cholesterol, the expression of the transduceosome proteins and the step of steroidogenesis carried out by the P450scc. Thus, progesterone improvement of mitochondrial structure and expression of fusion/fission molecules may contribute to the recovery of mitochondrial functions, including steroidogenesis.

In the proinflammatory environment caused by EAE induction, it was exciting that exogenous progesterone pretreatment preserved mRNA expression of mitochondrial proteins and neurosteroidogenic molecules (i.e., Star, VDAC and P450scc) and those of the endoplasmic reticulum (i.e., 5α-reductase, 3α-HSD and aromatase). Data from the present study have their counterpart in neurochemical studies showing that progesterone blocked the EAE-induced increase of the proinflammatory mediators tumor necrosis factor alpha (TNFα), its receptor TNFR1, the toll-like receptor 4 (TLR4) mRNAs, and increased mRNA expression of proteolipid protein (PLP) and myelin basic protein (MBP), the myelin transcription factors NKx2.2 and Olig1 and enhanced CC1+ oligodendrocyte density [35,55]. Former studies also demonstrated that progesterone decreased the number of Iba1+ microglial cells and the microglial marker CD11b, in addition to blocking proinflammatory factors in EAE and lysolecithin-induced demyelination [35,56]. Thus, the reduction of TSPO mRNA can be explained by progesterone inhibition of the reactive microgliosis. In neurodegeneration, which is also present in MS and EAE models, overexpression of TSPO is also a hallmark of reactive microglia [40].

The mechanism of action of progesterone in the EAE spinal cord is open to discussion. The role of the classical intracellular PR, membrane receptors sigma1, PRMC1 and GABA(A) receptors in neurosteroidogenesis was not addressed in the present experiments. However, binding of PR to the promoter sequence of P450scc, TSPO, 3β-HSD and aromatase was predicted by two programs (Alibaba 2.1 transcription factor binding prediction program, available at http://www.gene-regulation.com/pub/programs/alibaba2/index.html and Eukaryotic Promoter Database available at http://epd.vital-it.ch/), suggesting a transcription mechanism for these genes. Additionally, the PR-selective agonist Nestorone brings neuroprotection and anti-inflammatory effects to EAE mice, also suggesting a role for the classical receptor [42]. Increased levels of spinal cord DHP following progesterone treatment also point to the role of PR, as DHP is a ligand for this receptor [11]. The mentioned programs did not predict binding of PR to the promoters of Star, VDAC, 5α-reductase and 3α-HSD. Noorbakhsh et al. [18] have shown that EAE mice recover from the disease after receiving the reduced derivative THP, suggesting a GABAergic mechanism. In this context, the regulation of important neuronal functions by THP action on GABA-A receptors is well documented [57]. These findings suggest that progesterone effects are pleiotropic, employing genomic and membrane-initiated signaling mechanisms.

However, in the context of the present results, it should be recognized that measurement of mRNA levels of steroidogenic enzymes alone are not sufficient to assume that neurosteroid levels will be affected. Determination of the products of neurosteroidogenic enzymes by reliable methods such as mass spectrometry is needed to certify that the effects at the mRNA level are reflected in neurosteroid changes. In spite of this caveat, our data indicates that enhanced mRNA of neurosteroidogenic enzymes associates with the neuroprotective, promyelinating and anti-inflammatory effects already described in the spinal cord of EAE mice. Furthermore, progesterone protective effects on mitochondrial and endoplasmic reticulum may allow the recovery of neurosteroidododogenesis. In this case, endogenously synthesized neurosteroids may reinforce the protective effects of exogenous progesterone previously shown in MS mice.

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