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Research Report

Protective effects of progesterone administration on axonal pathology in mice with experimental autoimmune encephalomyelitis

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ABSTRACT

Experimental autoimmune encephalomyelitis (EAE), an induced model of Multiple Sclerosis presents spinal cord demyelination, axonal pathology and neuronal dysfunction. Previous work has shown that progesterone attenuated the clinical severity, demyelination and neuronal dysfunction of EAE mice (Garay et al., J. Steroid Biochem. Mol. Biol., 2008). Here we studied if progesterone also prevented axonal damage, a main cause of neurological disability. To this end, some axonal parameters were compared in EAE mice pretreated with progesterone a week before immunization with $MOG_{40–54}$ and in a group of steroid-free EAE mice. On day 16th after EAE induction, we determined in both groups and in control mice: a) axonal density in semithin sections of the spinal cord ventral funiculus; b) appearance of amyloid precursor protein (APP) immunopositive spheroids as an index of damaged axons; c) levels of the growth associated protein GAP43 mRNA and immunopositive cell bodies, as an index of aberrant axonal sprouting. Steroid-naive EAE mice showed decreased axonal density, shrunken axons, abundance of irregular vesicular structures, degenerating APP+ axons, increased expression of GAP43 mRNA and immunoreactive protein in motoneurons. Instead, EAE mice receiving progesterone treatment showed increased axonal counts, high proportion of small diameter axons, reduced APP+ profiles, and decreased GAP43 expression. In conclusion, progesterone enhanced axonal density, decreased axonal damage and prevented GAP43 hyperexpression in the spinal cord of EAE mice. Thus, progesterone also exerts protective effects on the axonal pathology developing in EAE mice.

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1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is an immune-mediated disease that exhibits characteristics in common with Multiple Sclerosis (MS) (Ayers et al., 2004; Kornek et al., 2006; Wujek et al., 2002). EAE can be induced in different species by administration of myelin proteins, including myelin oligodendrocyte glycoprotein (MOG) (Evron et al., 1984; Keith, 1978). The spinal cord of EAE mice presents low expression of the central myelin proteins MOG, myelin basic protein (MBP) and proteolipid protein (PLP). Neuropathology includes loss of axons, neuronal dysfunction, infiltration of inflammatory cells, microglial activation, astrocytosis, proliferation of oligodendrocyte precursor cells, and neurological deficits (Ayers et al., 2004; Garay et al., 2005; Kim et al., 2006; McQualter and Bernard, 2007; Sun et al., 2003).

A major contributing factor to clinical symptoms of MS and EAE is the axonal damage that worsens with disease progression (Blamire et al., 2007; Dutta and Trapp, 2007; Rovaris et al., 2005; Onuki et al., 2001; Wujek et al. 2002). Axonal pathology includes swelling, deficits in transport, degeneration and disruption of the normal axonal cytoarchitecture, changes of neurofilament phosphorylation, sodium channel distribution and development of amyloid precursor protein (APP) immunostaining (Ayers et al., 2004; Kim et al., 2006; Herrero-Herranz et al., 2008; Papadopoulos et al., 2006; Penkova and Hidalgo, 2003). There is a strong belief that restoration of myelin sheaths in EAE—a factor of critical importance for MS—will greatly preserve axonal survival (Zawadzka and Franklin, 2007). Therefore, testing neuroprotective agents that could preserve axonal integrity of EAE mice may be of potential applications in humans.

There is substantial background supporting a role for progesterone as a regulator of myelin protein expression and prevention of axonal damage both in the peripheral as well as the central nervous system (Azcoitia et al., 2003; De Nicola et al., 2006; Melcangi et al., 2005; Stein et al., 2008). When given to ovariectomized female and male animals, progesterone-treated rats show reduced axonal injury (seen via diminished APP immunoreactivity) when compared to controls (O'Connor et al., 2007). Therefore, it seems that under pathological circumstances or hormone deprivation, demyelination and axonal damage can be prevented by treatment or application of progesterone and/or its reduced derivatives.

Progesterone also induces strong immunosuppression during human pregnancy that may prevent relapses of MS (Confavreux et al., 1998; Druckmann and Druckmann, 2005; Hughes, 2004). This beneficial effect of pregnancy extends to several rodents species (Evron et al., 1984; Keith, 1978). Based on these demonstrations, the European multicentric trial POPART-MUS is currently enrolling post-partum women with MS who receive a mixture of estrogen/progestin to simulate steroid levels achieved during pregnancy, in an attempt to avoid the incidence of post-partum relapses (Confavreux et al., 1998; El-Etr et al., 2005). This clinical trial is supported by demonstrations that estrogens and progesterone protected and/or attenuate EAE development (Bebo et al., 2001; Elloso et al., 2005; Garay et al., 2007, 2008; Kim et al., 2006; Offner, 2004).

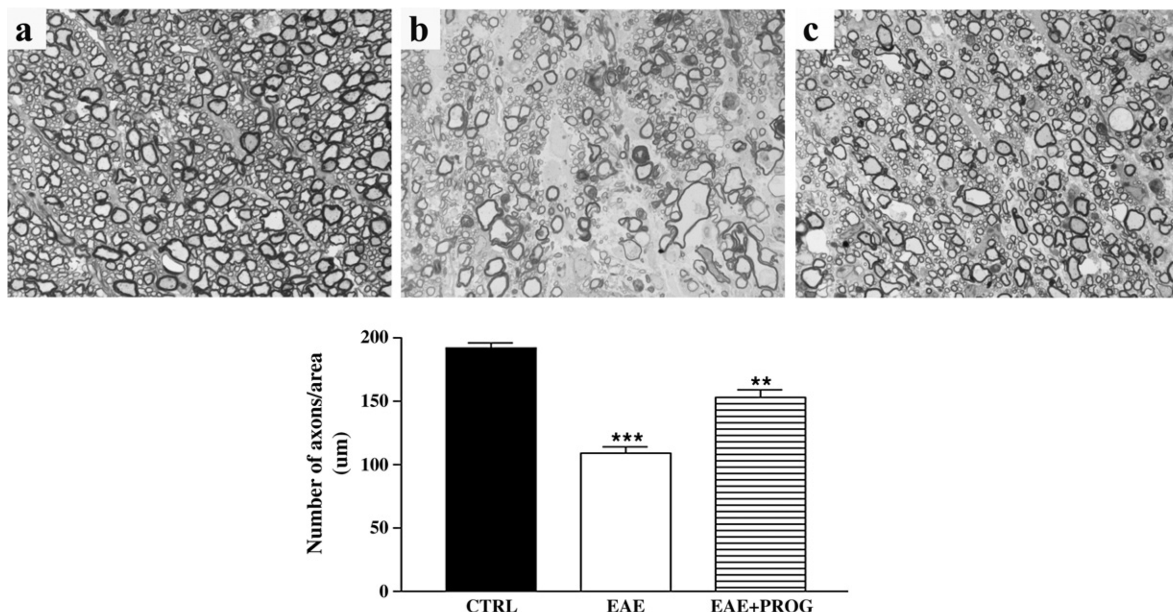

Fig. 1 – (Upper graph): representative photomicrographs of toluidine-blue stained semithin sections of the ventral white matter of the spinal cord in a control mouse (a), EAE (b) and EAE mouse receiving a 100 mg progesterone pellet a week before immunization with MOG $_{40–54}$ (c). Axonal abnormalities (reduced staining intensity, fewer axons, axonal swelling, and irregular vesicular structures) characterized EAE, whereas progesterone treatment attenuated these abnormalities. Lower graph: quantitative assessment of axonal density by computer-assisted analysis. The number of axons per unit area (μm^2) of the ventral funiculus was significantly lower in EAE respect of control mice (*$p<0.001$), whereas progesterone treatment significantly increased axonal counts (** EAE+ progesterone vs. EAE, $p<0.01$). ($n=3$ animals per group).**

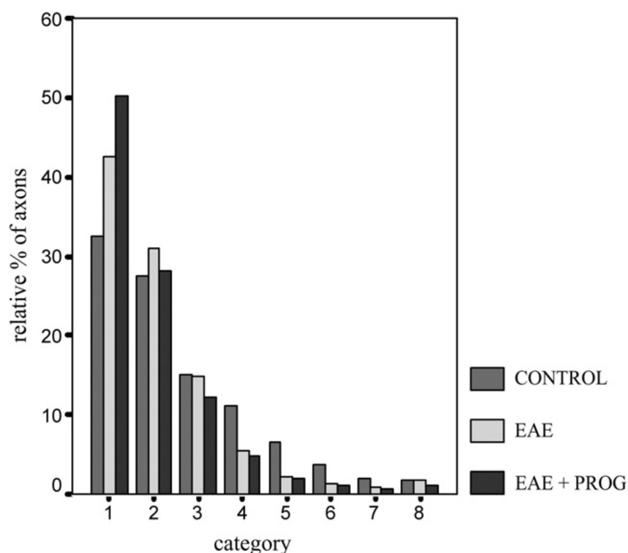

Fig. 2 – Distribution of axons according to diameter in control, EAE and EAE+ progesterone-treated mice. Axon diameters were divided in 8 categories, ranging from 0 to 5 μm (1), 5–10 (2), 11–15 (3), 16–20 (4), 21–25 (5), 26–30 (6), 31–35 (7) and >36 (8). A plot of % frequency against category demonstrated a prevalence of category 1 axons in the EAE+ progesterone-treated group.

Previous studies have shown that progesterone pre-treatment of EAE mice attenuated the clinical outcome, inflammatory cell infiltration and demyelination of the spinal cord (Garay et al., 2007, 2008). Here we studied if progesterone pre-treatment also prevented axonal pathology. Our experimental approach intended to simulate the inhibitory effects of high progestin levels of pregnancy on relapses of MS and the prevention of post-partum relapses by steroid administration.

2. Results

Toluidine-blue staining of spinal cord sections showed that axonal pathology was a prominent feature of mice with induced EAE, as expected from early reports (Ayers et al., 2004; Kim et al., 2006; Papadopoulos et al., 2006; Penkova and Hidalgo, 2003). Fig. 1 (upper photomicrographs) compared a typical control mouse (a), EAE mouse (b) and EAE mouse receiving progesterone (c). A prominent axonal loss occurred in EAE mice, which showed areas of reduced staining intensity, shrunken axons and abundant vesicular structures of irregular shape and size (Fig. 1b). The vesicular structures may conform to spheroids resulting from disruption of axonal transport or end bulbs caused by Wallerian degeneration (Ayers et al., 2004; Coleman, 2005). In contrast, EAE mice receiving progesterone (Fig. 1c) showed increased axonal density, increased staining intensity, and fewer swollen axons or vesicular structures. These observations were substantiated by quantitative analysis of axon density in the ventral white matter. Fig. 1 (lower graph) shows that axonal counts were significantly reduced in EAE mice (108.9 ± 5.4 axons per μm2 vs. control: 191.8 ± 4; $p < 0.001$), whereas progesterone treatment increased this parameter (EAE plus progesterone: 152.9 ± 5.9, $p < 0.01$ vs. EAE). Although progesterone enhanced axonal counts, their number was still lower than control ($p < 0.01$). On closer inspection, it appeared that several axons in the EAE + progesterone group showed a reduced diameter in comparison to control axons (Fig. 1a). Fig. 2 shows the relationship between axon numbers vs.

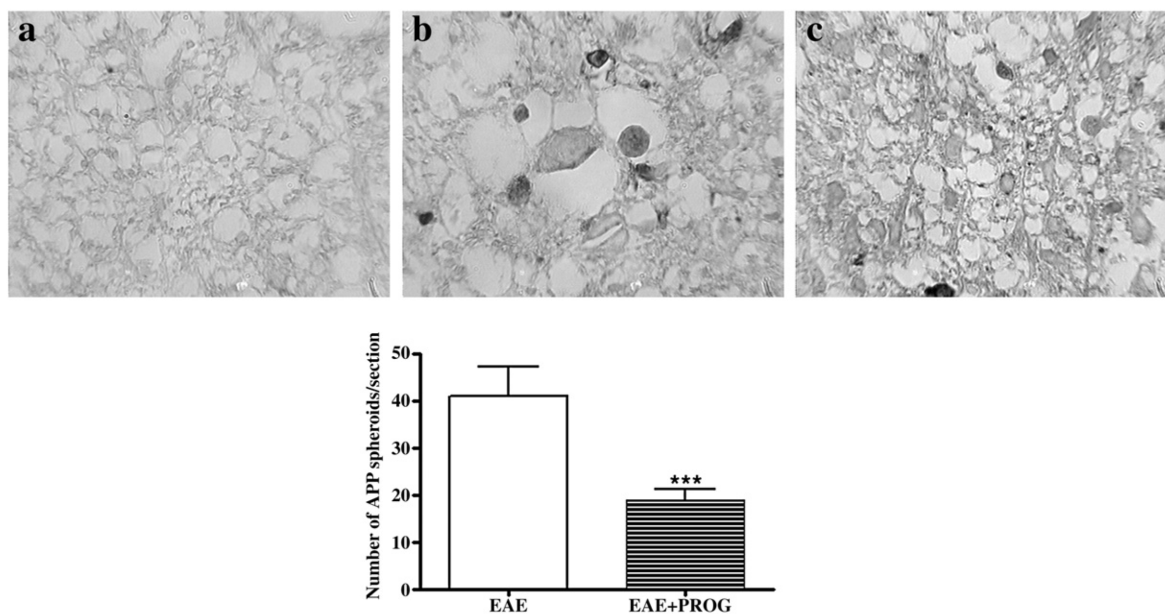

Fig. 3 – (Upper graph): the abundance of APP immunostaining of degenerating axons in EAE mouse (b), contrasts with lack of APP immunoreactivity in control mouse (a) and with greatly diminished APP profiles in EAE mouse receiving progesterone (c). Lower graph: quantitative analysis of the number of APP+ degenerating axons between EAE mice with or without steroid treatment, demonstrated a significant progesterone effect (* $p < 0.001$; $n = 10$ animals per group).**

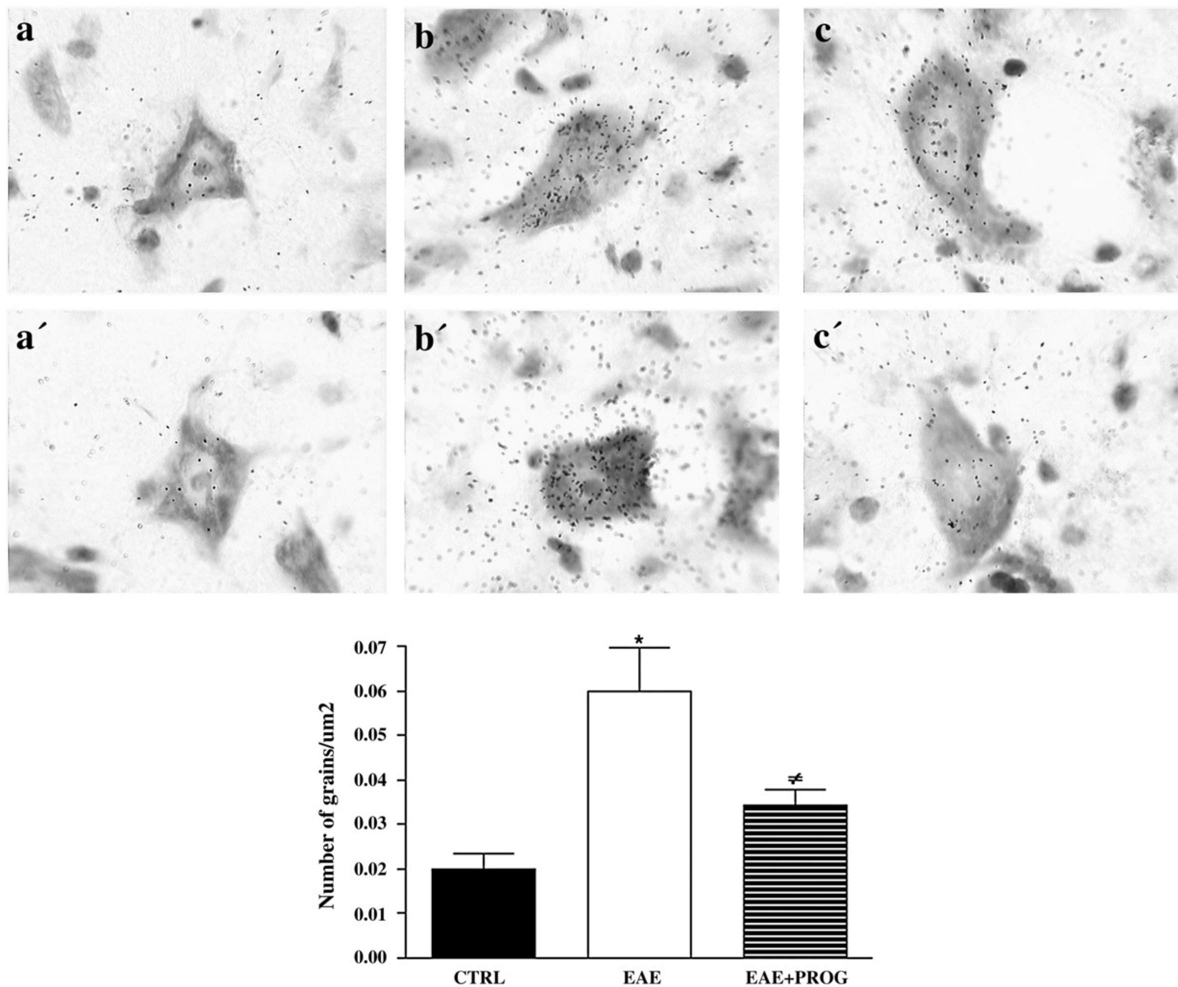

Fig. 4 – (Upper graph): number of grains per unit area (μm^2) representing labelled oligonucleotide probe hybridized to GAP43 mRNA in control mice (a, a′), EAE (b, b′) and EAE mice receiving progesterone (c, c′). EAE shows increased grain density respect of both control and EAE+ progesterone mice. Lower graph: upon quantitative grain counting, EAE mice showed a 2.7 fold increase respect of controls ($^*p<0.05$). Hyperexpression of GAP mRNA was decreased by progesterone treatment (EAE vs. EAE+ progesterone: $^‡p<0.05$; $n=5$–11 animals per group).

axonal diameter, measured by computer-assisted image analysis. It was observed that group differences (control, EAE and EAE+progesterone) were most obvious in the smaller range (1–5 μm), pointing out that small calibre axons predominated in the progesterone-treated EAE mice.

In order to corroborate the high incidence of axonal degeneration in EAE mice (Blamire et al., 2007; Dutta and Trapp, 2007; Rovaris et al., 2005; Onuki et al., 2001; Wujek et al. 2002) we used immunostaining for APP. APP deposits appear in axons after focal blockage of axonal transport and are also

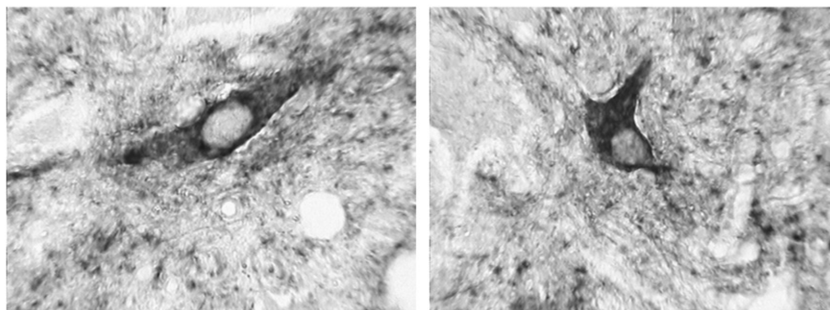

Fig. 5 – GAP43 immunopositive motoneurons in EAE mice spinal cord. These cells were detected in two out of 5 EAE mice, whereas none were observed in seven EAE mice receiving progesterone. Magnification ×1000.

present in spheroids (Ayers et al., 2004; Kim et al., 2006; Papadopoulos et al., 2006; Penkova and Hidalgo, 2003). This EAE-associated axon damage may occur by a mechanism similar to Wallerian degeneration (rev. in Coleman, 2005). As expected, APP immunostaining was absent in control tissues (Fig. 3a), whereas APP-positive spheroids of variable sizes were detected in lumbar spinal cord sections from EAE mice (Fig. 3b). In contrast, these APP+ profiles were reduced in progesterone-treated EAE mice (Fig. 3c). Light microscope observations were supported by quantitative analysis (Fig. 3, lower graph), which revealed that progesterone administration to EAE mice reduced by half the number of APP-stained profiles appearing in steroid-naive EAE mice (EAE: 40.84±6.31 APP+ profiles per section; EAE plus progesterone: 18.81±2.29, $p<0.001$ vs. EAE; $n=10$ animals per group).

Further proof for a progesterone effect on axons of EAE mice was provided by determination of GAP43 mRNA. This molecule was poorly expressed in controls (Fig. 4 a, a′), since few grain deposits were observed in perikarya from normal motoneurons. However, probe hybridized to GAP43 mRNA experienced a 3.2-fold increase in motoneurons from steroid-naive EAE mice, measured as number of grains deposited over neuronal body per area (EAE: 0.0599±0.009 grains/μm^2 vs. control: 0.02±0.003; $p<0.05$) (Fig. 4 b, b′ and lower graph). Progesterone treatment significantly reduced the GAP43 mRNA hyperexpression in motoneurons of EAE mice (Fig. 4 c, c′ and lower graph), such as that after steroid therapy, GAP43 mRNA levels of progesterone-treated EAE mice was no longer different from control levels (EAE plus progesterone: 0.034±0.0036; $p<0.05$ vs. EAE; NS vs. control). Few GAP43 immunopositive motoneurons were occasionally observed in two out of five spinal cord sections from steroid-naive EAE mice (Fig. 5), but they were absent in the ventral horns of control and progesterone-treated EAE mice ($n=5$–11 animals per group).

3. Discussion

The present investigation shows that progesterone pretreatment enhanced axonal density, decreased axonal damage and prevented GAP43 hyperexpression in the spinal cord of EAE female mice. The use of females was based on demonstrations that immune reactivity is greater in females than males and that men are less susceptible to the disease than women (Bebo et al., 2001; Confavreux et al., 1998; El-Etr et al., 2005). Our paradigm simulates the situation in pregnant women with MS, in which relapses are prevented if patients are pretreated with steroids immediately post-partum. From the clinical perspective, providing steroid treatment after disease onset may be more relevant for men and non-pregnant women suffering from MS. Further experiments should unveil whether progesterone induces axonoprotection if given at the initiation or after disease onset in EAE mice as well as in other demyelination models. Thus, axonal data are in concert with previous results showing that progesterone stimulates myelination and enhances neuronal parameters of EAE mice (Garay et al., 2007, 2008), suggesting that restoration of myelin sheaths preserves axonal survival (Zawadzka and Franklin, 2007).

In rodents with induced EAE, inflammatory attack causes early degeneration and loss of myelinated axons (Dutta and Trapp, 2007; Herrero-Herranz et al. 2008; Kim et al., 2006; Kornek et al., 2000; Onuki et al., 2001; Papadopoulos et al., 2006; Russel et al., 2006; Wujek et al., 2002). Mechanisms leading to axonal pathology include oxidative stress, nitric oxide, Na,K-ATPase dysfunction, glutamatergic excitotoxicity, and loss of myelin protection (Dutta and Trapp, 2007; Penkowa and Hidalgo, 2003; Giardino et al., 2004; Werner et al., 2000). Recent work also indicates that EAE outcome depends on axonal recovery and axonoprotection (Diem et al., 2007; Herrero-Herranz et al., 2008). Since axonal damage of EAE and MS becomes more widespread as disease advances (Dutta and Trapp, 2007; Kim et al., 2006; Onuki et al., 2001) axonoprotection becomes a fundamental goal for demyelinating diseases. Among other factors, sex steroid hormones have shown this property (Bebo et al., 2001; Elliott et al., 1997; Elloso et al., 2005; Morales et al., 2006; Offner, 2004; Palazynski et al., 2004).

We already reported that progesterone prevented the loss of myelin in specific foci of the white matter of EAE mice and increases the expression of MBP and PLP (Garay et al., 2007). This effect may act in conjunction with progesterone's ability to protect axons from EAE mice. Thus, progesterone-treated EAE mice showed increased axonal density, disappearance of vesicular structures and spheroids, and reduced number of APP+ damaged axons. In view of the proposed etiopathogenic mechanisms that generate axonal injury in EAE, it is important to recall progesterone's ability to modulate these parameters in the nervous system. Thus, progesterone shows antioxidant and antiglutamatergic activity, restores the deficient expression of Na,K-ATPase, exerts anti-inflammatory, immunomodulatory and protective effects in trauma and neurodegeneration and down-regulates the APP immunoreactivity of injured axons (Garay et al., 2007; Gonzalez Deniselle et al., 2005; Labombarda et al., 2002; O'Connor et al., 2007; Ogata et al., 1992; Pettus et al., 2005; Stein, 2008). Finally, the promyelinating effects of progesterone may reassure a normal saltatory conduction and neurotransmission (Azcoitia et al., 2003; Labombarda et al., 2009; Melcangi et al., 2007; Schumacher et al., 2008).

The present work also demonstrated that axonal damage was accompanied by hyperexpression of GAP43 mRNA, which normalized in progesterone-treated EAE mice. Few GAP43+ cells appeared in some steroid-naive mice, indicating that EAE's strongest effect took place at the mRNA level. GAP43 is expressed during embryogenesis and up-regulates in neurons after injury and degeneration (Benowitz and Routtenberg, 1997; Curtis et al., 1993; González Deniselle et al., 1999; Labombarda et al., 2002) and in astrocytes from Alzheimer's patients (De la Monte et al., 1995). The up-regulation of GAP-43 might represent an exaggerated response to neurodegeneration and denervation, but may also be detrimental, because abnormal synaptic reorganization results from GAP-43 induced sprouting (Benowitz and Routtenberg, 1997) and regions with high GAP-43 expression show poor myelination (Kapfhammer and Schwab, 1994). We hypothesized that attenuation of GAP-43 mRNA hyperexpression by progesterone stops an exaggerated neuronal response to axonal loss and degeneration and could stimulate remyelination.

As postulated for other conditions (De Nicola et al., 2006; Schumacher et al., 2008), multiple mechanisms may explain progesterone actions in EAE mice. These mechanisms range from genomic effects due to intracellular progesterone receptors (PR), to non-genomic effects caused by membrane or axonal PRs (Schumacher et al., 2008; Waters et al., 2008), Other possibilities for progesterone effects in EAE mice may involve the reduced metabolites 3 α-hydroxy (dihydroprogesterone) and 3α,5α-tetrahydroxyprogesterone (allopregnanolone), which are also endowed with neuroprotective and myelinating properties (Azcoitia et al., 2003; Ciriza et al., 2004).

Besides having direct effects on the spinal cord, progesterone is a recognized immunomodulatory factor. Increased progesterone levels during pregnancy modulates the immune system, changing a Th_1 pro-inflammatory response into a Th_2 anti-inflammatory reaction (Druckmann and Druckmann, 2005; Hughes, 2004). Activated lymphocytes exposed to progesterone secrete the non-inflammatory cytokines IL_3, IL_4, IL_{10} and reduce the inflammatory cytokines IFN-γ, TNFα and IL_2 (Druckmann and Druckmann, 2005). Immunomodulatory effects of progesterone are extended beyond pregnancy, as they have been shown following traumatic brain injury (Stein, 2008). In order to account for the beneficial effect of estrogens in EAE, Offner (2004) proposed that steroids inhibit encephalitogenic T cells and the migration of immune cells into the CNS. Thus, progesterone may prevent immune-mediated axonal damage, besides its direct protective effects on axons in EAE mice.

Progesterone effects on glial cells may be also involved in axonoprotection. In this respect, it is worth recalling that the spinal cord of EAE mice shows a pronounced astrogliosis, with strong expression of the glial fibrillary acidic protein (GFAP) (Garay et al., 2005). Astrocyte activation imposes a barrier to axonal growth and it is source of pro-inflammatory factors in EAE models (Ayers et al., 2004). Preliminary results demonstrated that progesterone pre-treatment of EAE mice down regulated the number of GFAP+ cells. Thus, progesterone's effects on astrocytes could contribute to axonal recovery. Another glial cell type involved in axonal recovery is the oligodendrocyte, which suffers apoptotic cell death in EAE and MS. It is possible that replenishment of oligodendrocyte lineage cells under the influence of progesterone (Labombarda et al., 2009) contributed to axonoprotection, since myelin-forming cells render trophic support for axon survival Dutta and Trapp, 2007). These possibilities suggest involvement of several cell types in progesterone effects in EAE mice.

At the clinical level, the POPART-MUS study relies on the protective effects of estrogens and progesterone for the prevention of MS relapses (Confavreux et al., 1998). Previous data has demonstrated the usefulness of progesterone to stimulate remyelination and neuronal parameters, to decrease immune cell infiltration and attenuate neurological signs of EAE mice (Garay et al., 2007, 2008). Presently, the range of progesterone effects was extended to the prevention of axonal loss, axonal degeneration and on the expression of a molecule associated to aberrant axonal sprouting. Whereas additional markers may be needed to firmly establish progesterone reversal of axonal pathology, it was highly rewarding that at least four markers (i.e., APP immunostaining, GAP43 expression, axonal counts and measurement of axonal diameter) showed changes following hormone treatment. It is hoped that results obtained in EAE mice may disclose if progesterone brings beneficial effects to pregnant women with MS.

4. Experimental procedures

4.1. *EAE induction*

Adult female C57Bl/6 mice were immunized using a $MOG_{40–54}$ peptide (200 μg per mouse) emulsified in complete Freund's adjuvant (Sigma, St Louis. MO) containing 0.6 mg *Mycobacterium tuberculosis* (Instituto Malbran, Argentina). The animals also received i.p. injections of Pertussis toxin (400 ng) (Sigma, St Louis) immediately after the immunization and another boost on the day after. Control mice received the emulsion without the $MOG_{40–54}$ peptide and the Pertussis toxin. The animals developed EAE approximately on day 10 and they were sacrificed on day 17 when the disease was still in an acute phase. One group of EAE mice remained untreated and another group received sc under the skin of the neck a single 100 mg pellet of progesterone (Sigma) 1 week before EAE induction. Compressed pellets of progesterone crystals were made using a manual pellet press and used immediately without storage. In our hands, progesterone pellet implantation is a reliable method to study progesterone neuroprotection in mice (Gonzalez Deniselle et al., 2005). At the time of sacrifice, plasma progesterone levels in the progesterone-treated group reached ~80 ng/ml (Garay et al., 2007). These levels resemble those achieved by the mouse on days 15–16 of pregnancy (Holinka et al., 1979). Vaginal smears revealed that all EAE mice were in metestrus at the time of killing, resembling the cytology of pregnant mice. The effects of progesterone on cell infiltration and demyelination of the spinal cord of EAE mice were already published (Garay et al., 2007). Clinically, we have shown that in EAE mice disease onset, which in hormone-free mice averaged ≈9.9 days, was delayed by implantation of a progesterone pellet. Additionally, peak score was significantly reduced in EAE mice under hormone treatment. Thus, the focus of the present report was on the axonal pathology of EAE mice. Animal procedures followed the NIH Guide for the Care and Use of Laboratory Animals (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 keep the number of mice to a minimum.

4.2. *Determination of axon density in semithin sections*

EAE mice with or without progesterone treatment, as well as control animals were anesthetized and perfused transcardially with a solution containing 4% paraformaldehyde (PFA) plus 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.4. Lumbar spinal cords were removed and small blocks of tissue were obtained by cutting transverse sections of 2–3 mm maximum length. Blocks were immersed for 3 h in the same fixative. After overnight washing in 0.1 M sodium phosphate buffer, tissue blocks containing the lumbar ventral horns were postfixed in 1% OsO_4 in 0.1 M phosphate buffer pH 7.4 for 1 h

and exposed to 1% uranyl acetate. Afterwards, tissue blocks were dehydrated and flat-embedded in Durcupan (Fluka Chemic AG, Sweden). Semithin sections (1 μm) were stained with toluidine blue. In order to quantify axonal loss, we followed a line sampling method similar to that described by Olby and Blakemore (1996). Images of toluidine blue stained 1 μm-thick cross-sections corresponding to the ventral funiculus of the lumbar spinal cord were captured with a 60× objective using a computer-assisted image analysis equipped with a video camera (Bioscan OptimasVI). Six tissue strips extending from the gray matter to the pial surface were drawn following a grid superimposed to the captured image. The strips were positioned at a fixed distance to the left and right of a central line in the central of the ventral funiculus. All axons regardless of the myelination state intercepted by a sampling line were counted and the cross-sectional area was manually outlined and automatically measured. Due to the limitation in resolution, fibers of cross-sectional area smaller than 1 μm^2 were not included in the counts. Axons were counted in 6 strips per section (~1200 axons in controls animals), for a total of 4 sections per animal ($n=3$ animals per experimental group). Results were expressed as number of axons per μm^2 (mean±S.E.M.).

4.3. *APP staining of degenerating axons*

For determination of APP protein immunostaining, the method of Linker et al. (2005) was followed. To this purpose, groups of EAE mice with and without progesterone treatment and controls were transcardially perfused with 4% PFA and sections of their spinal cords embedded in paraffin. 5 μm slices of the spinal cord obtained with a conventional microtome were first incubated with 3% H_2O_2 in methanol to block endogenous peroxidase. For antigen retrieval, slices were microwaved for 5″ and then preincubated in 10% BSA. For immunocytochemistry we employed a 1/1000 dilution of an anti-APP monoclonal antibody (Clone 22C11, Chemicon) in PBS containing 10% BSA during 20 h at 4 °C. As second antibody, we used a biotin-conjugated goat antimouse IgG (diluted 1/200 in 1% BSA). Slices were further processed according to the ABC kit instructions (Vector Labs, CA, USA) and developed with 3,3′-diaminobenzidine (DAB) 0.5 mg/ml, 0.05% H_2O_2. Sections were counterstained with cresyl violet, dehydrated, cleared and mounted with Permount (Fisher Chemical, USA). Nonspecific staining was assessed in the absence of primary antibody. The number of axonal spheroids showing APP staining was determined in the dorsal, lateral and ventral funiculus of the white matter using a 40× objective. Five to 8 sections of the spinal cord per animal were analyzed to determine the number of APP+ degenerating axons using computed-assisted image analysis ($n=10$ animals per experimental group). Data were expressed as number of APP+ profiles per section (mean± S.E.M.).

4.4. *In situ hybridization (ISH) of GAP43 mRNA and immunocytochemical determination of GAP43 protein*

ISH for GAP43 mRNA was carried out following a previously published protocol (González Deniselle et al., 1999). A synthetic oligonucleotide probe with the sequence 5′-CGCAGCCTTATGAGCCTTATCTTCCGGCTTGACACCATC-3′ (Elliott et al., 1997) was end labelled with (^{35}S)-ATP using the enzyme terminal transferase. For ISH, groups of EAE ($n=11$), EAE+ progesterone ($n=9$) and control mice ($n=5$) were transcardially perfused with 4% PFA prepared in diethylpirocarbonate-treated water. Cryostat sections of cervical spinal cords were hybridized at 37 °C with 8×10^6 c.p.m. ^{35}S-labelled probe/ml in SSC buffer containing 0.02% Ficoll 400, 0.02% polyvinylpirrolidone, 0.02% BSA, 50% formamide, 3× SSC buffer, 10 mM dithiothreitol, 0.1 mg/ml salmon sperm DNA, 1 mM EDTA, 4 μg/ml heparin, 0.4 mg/ml tRNA, and 10% dextran sulfate. After hybridization, sections were washed several times in SSC, dried, dipped into Kodak NTB-2 emulsion and exposed for 2 weeks. Afterwards, sections were developed with Dektol (Kodak Dektol, 1:2 dilution with water), fixed, counterstained with cresyl violet—to visualize neuronal soma—and coverslipped with Permount. Quantitative grain counting (number of grains over cresyl-violet stained neuronal bodies minus background/μm^2) was performed by a computerized image analysis system (Bioscan Optimas, Edmonton, WA, USA) equipped with a VT-C33ON video camera. Results were expressed as the mean grain density/μm^2±S.E.M.

GAP43 immunocytochemistry was carried out in paraffin sections of the spinal cord, using a previously described protocol (González Deniselle et al., 1999). A polyclonal anti-GAP43 antiserum (gift of G. Wilkin, Imperial College of Science, Technology and Medicine, London, UK) was used at 1/1000 dilution. Specificity and characteristics of this antibody were already published (Curtis et al., 1993). The first antibody was revealed using a biotinylated IgG goat anti-rabbit secondary antibody (ABC Kit, Vector), with final reaction employing 2.5% nickel-intensified 0.025% DAB in 0.01% H_2O_2. The spinal cord ventral horn was screened for the presence of GAP43+ neurons in five EAE and seven EAE+ progesterone-treated mice.

4.5. *Statistical analysis*

Slides were coded before quantitative analysis by experimenters blind to the groups of mice analyzed. For statistical analysis we used Graph Pad PRISM 4 software. In the case of axonal number and GAP43 expression, in which more than 3 groups were compared, we used one-way ANOVA followed by the Newman–Keuls post-hoc test. When only two groups were compared (APP immunoreactive profiles) we used the Student's t test. A p value of less than 0.05 was considered significant.

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REFERENCES

Ayers, M.M., Hazelwood, L.J., Catmull, D.V., Wang, D., McKormack, Q., Bernard, C.C., Orian, J.M., 2004. Early glial responses in murine models of multiple sclerosis. Neurochem. Int. 45, 409–419.

Azcoitia, I., Leonelli, E., Magnaghi, V., Veiga, S., Garcia Segura, L.M., Melcangi, R., 2003. Progesterone and its derivatives dihydroprogesterone and tetrahydroprogesterone reduce myelin fiber morphological abnormalities and myelin fiber loss in the sciatic nerve of aged rats. Neurobiol. Aging 24, 853–860.

Bebo, B.F., Fyfe-Johnson, A., Adlard, K., Beam, A.G., Vandenbark, A.A., Offner, H., 2001. Low-dose estrogen therapy ameliorates experimental autoimmune encephalomyelitis in two different inbred mouse strains. J. Immunol. 166, 2080–2089.

Benowitz, L.I., Routtenberg, A., 1997. GAP-43: an intrinsic determinant of neuronal development and plasticity. Trends in Neurosci. 20, 84–91.

Blamire, A.M., Cader, S., Lee, M., Palace, J., Matthews, P.M., 2007. Axonal damage in the spinal cord of multiple sclerosis patients detected by magnetic resonance spectroscopy. Magn. Reson. Med. 58, 880–885.

Ciriza, I., Azcoitia, I., Garcia-Segura, L.M., 2004. Reduced progesterone metabolites protect rat hippocampal neurones from kainic acid excitotoxicity in vivo. J. Neuroendocrinol. 16, 58–63.

Coleman, M., 2005. Axon degeneration mechanisms: commonality amid diversity. Nat. Rev. Neurosci. 6, 889–898.

Confavreux, C., Hutchinson, M., Hours, M.M., Cortinovis-Tourniaire, P., Moreau, T., 1998. Rate of pregnancy-related relapse in multiple sclerosis. New Engl. J. Med. 339, 285–291.

Curtis, R., Green, D., Lindsay, R.M., Wilkin, G.P., 1993. Up-regulation of GAP-43 and growth of axons in rat spinal cord after compression. J. Neurocytol. 22, 51–64.

De la Monte, S.M., Ng, S.-C., Hsu, D.W., 1995. Aberrant GAP-43 gene expression in Alzheimer's disease. Am. J. Pathol. 147, 934–946.

De Nicola, A.F., Gonzalez, S.L., Labombarda, F., Gonzalez Deniselle, M.C., Garay, L., Guennoun, R., Schumacher, M., 2006. Progesterone treatment of spinal cord injury: effects on receptors, neurotrophins, and myelination. J. Mol. Neurosci. 28, 3–15.

Diem, R., Sättler, M.B., Bähr, M., 2007. Neurodegeneration and -protection in autoimmune CNS inflammation. J. Neuroimmunol. 184, 27–36.

Druckmann, R., Druckmann, M.A., 2005. Progesterone and the immunology of pregnancy. J. Steroid Biochem. Mol. Biol. 97, 389–396.

Dutta, R., Trapp, B.D., 2007. Pathogenesis of axonal and neuronal damage in multiple sclerosis. Neurology 68 (Suppl. 3), S22–S31.

El-Etr, M., Vukusic, S., Gignoux, L., Durand-Dubief, F., Achiti, I., Baulieu, E.E., Confavreux, C., 2005. Steroid hormones in multiple sclerosis. J. Neurol. Sci. 233, 49–54.

Elliott, E.J., Parks, D.A., Fishman, P.S., 1997. Effect of proximal axotomy on GAP-43 expression in cortical neurons in the mouse. Brain Res. 755, 221–228.

Elloso, M.M., Phiel, K., Henderson, R.A., Harris, H.A., Adelman, S.J., 2005. Suppression of experimental autoimmune encephalomyelitis using estrogen receptor-selective ligands. J. Endocrinol. 185, 243–244.

Evron, S., Brenner, T., Abramsky, O., 1984. Suppressive effect of pregnancy on the development of experimental allergic encephalomyelitis in rabbits. Am. J. Reprod. Immunol. 5, 109–113.

Garay, L., González Deniselle, M.C., Villa, A.M., Garcea, O., Sica, R.E.P., De Nicola, A.F., 2005. Immunohistopathological changes in myelin oligodendrocyte glycoprotein 40–54 induced experimental autoimmune encephalomyelitis. Multiple Sclerosis 11, 43–52.

Garay, L., Gonzalez Deniselle, M.C., Lima, A., Roig, P., De Nicola, A.F., 2007. Effects of progesterone in the spinal cord of a mouse model of multiple sclerosis. J. Steroid Biochem. Mol. Biol. 107, 228–237.

Garay, L., Gonzalez Deniselle, M.C., Gierman, L., Meyer, M., Lima, A., Roig, P., De Nicola, A.F., 2008. Steroid protection in the experimental autoimmune encephalomyelitis model of multiple sclerosis. Neuroimmunomodulation 15, 76–83.

Giardino, L, Giuliani, A., Fernandez, M., Calzà, L., 2004. Spinal motoneurone distress during experimental allergic encephalomyelitis. Neuropathol. Appl. Neurobiol. 30, 522–531.

González Deniselle, M.C., Grillo, C., González, S., Roig, P., De Nicola, A.F., 1999. Evidence for down-regulation of GAP-43 mRNA in Wobbler mouse spinal motoneurons by corticosterone and a 21-aminosteroid. Brain Res. 841, 78–84.

Gonzalez Deniselle, M.C., Garay, L., Gonzalez, S., Guennoun, R., Schumacher, M., De Nicola, A.F., 2005. Progesterone restores retrograde labeling of cervical motoneurons in Wobbler mouse motoneuron disease. Exp. Neurol. 195, 518–523.

Herrero-Herranz, E., Pardo, L.A., Gold, R., Linker, R.A., 2008. Pattern of axonal injury in murine myelin oligodendrocyte glycoprotein induced experimental autoimmune encephalomyelitis: implications for multiple sclerosis. Neurobiol. Dis. 30, 162–173.

Holinka, C.F., Tseng, Y.C., Finch, C.E., 1979. Reproductive aging in C57BL/6J mice: plasma progesterone, viable embryos and resorption frequency throughout pregnancy. Biol. Reprod. 20, 1201–1211.

Hughes, M.D., 2004. Multiple sclerosis and pregnancy. Neurol. Clin. 22, 757–769.

Kapfhammer, J.P., Schwab, M.E., 1994. Inverse patterns of myelination and GAP-43 expression in the dault CNS: neurite growth inhibitors as regulators of neuronal plasticity? J. Comp. Neurol. 340, 194–206.

Keith, A.B., 1978. Effect of pregnancy on experimental allergic encephalomyelitis in guinea pigs and rats. J. Neurol. Sci. 38, 317–326.

Kim, J.H., Budde, M.D., Liang, H.F., Klein, R.S., Russell, J.H., Cross, A.H., Song, S.K., 2006. Detecting axon damage in spinal cord from a mouse model of multiple sclerosis. Neurobiol. Dis. 2, 626–632.

Kornek, B., Storch, M.K., Weissert, R., Wallstroem, E., Stefferl, A., Olsson, T., Linington, C., Schmidbauer, M., Lassmann, H., 2000. Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. Am. J. Pathol. 157, 267–276.

Labombarda, F., Gonzalez, S.L., Gonzalez, D.M., Guennoun, R., Schumacher, M., De Nicola, A.F., 2002. Cellular basis for progesterone neuroprotection in the injured spinal cord. J. Neurotrauma 19, 343–355.

Labombarda, F., Gonzalez, S.L., Lima, A., Roig, P., Guennoun, R., Schumacher, M., De Nicola, A.F., 2009. Effects of Progesterone on oligodendrocyte progenitors, oligodendrocyte transcription factors and myelin proteins following spinal cord injury. Glia. 57 (8), 884–897.

Linker, R.A., Sendtner, M., Gold, R., 2005. Mechanisms of axonal degeneration in EAE—lessons from CNTF and MHC I knockout mice. J. Neurol. Sci. 233, 167–172.

McQualter, J.L., Bernard, C.C., 2007. Multiple sclerosis: a battle between destruction and repair. J. Neurochem. 100, 295–306.

Melcangi, R.C., Cavarretta, I.T., Ballabio, M., Leonelli, E., Schenone, A., Azcoitia, I., Garcia-Segura, L.M., Magnaghi, V., 2005. Peripheral nerves: a target for the action of neuroactive steroids. Brain Res. Brain Res. Rev. 48, 328–338.

Morales, L.B.J., Loo, K.K., Liu, H., Peterson, Tiwari-Woodruff, C.S., Voskuhl, R.R., 2006. Treatment with an estrogen receptor ligand is neuroprotective in experimental autoimmune encephalomyelitis. J. Neurosci. 26, 6823–6833.
O'Connor, C.A., Cernak, I., Johnson, Vink, R., 2007. Effects of progesterone on neurologic and morphologic outcome following diffuse traumatic brain injury in rats. Exp. Neurol. 205, 145–153.
Offner, H., 2004. Neuroimmunoprotective effects of estrogen and derivatives in experimental autoimmune encephalomyelitis: therapeutic implications for multiple sclerosis. J. Neurosci. Res. 78, 603–624.
Ogata, T., Nakamura, Y., Tsuji, K., Shibata, T., Kataoka, K., 1992. Steroid hormones protect spinal cord neurons from glutamate toxicity. Neurosci. 55, 445–449.
Olby, N.J., Blakemore, W.F., 1996. Primary demyelination and regeneration of ascending axons in the dorsal funiculus of the rat spinal cord following photochemically induced injury. J. Neurocytol. 25, 465–480.
Onuki, M., Ayers, M.M., Bernard, C.C., Orian, J.M., 2001. Axonal degeneration is an early pathological feature in autoimmune-mediated demyelination in mice. Microsc. Res. Tech. 152, 731–739.
Palazynski, K., Liu, H., Loo, K., Voskuhl, R.R., 2004. Estriol treatment ameliorates disease in males with experimental autoimmune encephalomyelitis: implications for multiple sclerosis. J. Neuroimmunol. 149, 84–89.
Papadopoulos, D., Pham-Dinh, D., Reynolds, R., 2006. Axon loss is responsible for chronic neurological deficit following inflammatory demyelination in the rat. Exp. Neurol. 197, 373–385.
Penkowa, M., Hidalgo, J., 2003. Treatment with metallothionein prevents demyelination and axonal damage and increases oligodendrocyte precursors and tissue repair during experimental autoimmune encephalomyelitis. J. Neurosci. Res. 72, 574–586.
Pettus, E.H., Wright, D.W., Stein, D.G., Hoffman, S.W., 2005. Progesterone treatment inhibits the inflammatory agents that accompany traumatic brain injury. Brain Res. 1049, 112–119.
Rovaris, M., Gallo, A., Falini, A., Benedetti, B., Rossi, P., Comola, M., Scotti, G., Comi, G., Filippi, M., 2005. Axonal injury and overall tissue loss are not related in primary progressive multiple sclerosis. Arch. Neurol. 62, 898–902.
Russell, J.H., Cross, A.H., Song, S.K., 2006. Detecting axon damage in spinal cord from a mouse model of multiple sclerosis. Neurobiol. Dis. 21, 626–632.
Schumacher, M., Sitruk-Ware, R., De Nicola, A.F. 2008. Progesterone and progestins: neuroprotection and myelin repair. Curr. Opin. Pharmacol. 8, 740–746.
Stein, D.G., 2008. Progesterone exerts neuroprotective effects after brain injury. Brain Res. Rev. 57, 386–397.
Sun, D., Zhang, Y., Wei, B., Peiper, S.C., Shao, H., Kaplan, H.J., 2003. Encephalitogenic activity of truncated myelin oligodendrocyte glycoprotein (MOG) peptides and their recognition by CD8+ MOG-specific T cells on oligomeric MHC class I molecules. Int. Immunol. 15, 261–268.
Waters, E.M., Torres-Reveron, A., McEwen, B.S., Milner, T.A., 2008. Ultrastructural localization of extranuclear progestin receptors in the rat hippocampal formation. J. Comp. Neurol. 511, 34–46.
Werner, P., Pitt, D., Raine, C.S., 2000. Glutamate excitotoxicity—a mechanism for axonal damage and oligodendrocyte death in multiple sclerosis? J. Neural. Transm. Suppl. 60, 375–385.
Wujek, R., Bjartmar, C., Richer, E., Ransohoff, R.M., Yu, M., Tuohy, V.K., Trapp, B.D., 2002. Axon loss in the spinal cord determines permanent neurological disability in an animal model of multiple sclerosis. J. Neuropathol. Exp. Neurol. 61, 23–32.
Zawadzka, M., Franklin, R.J., 2007. Myelin regeneration in demyelinating disorders: new developments in biology and clinical pathology. Curr. Opin. Neurol. 20, 294–298.