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Steroid Protection in the Experimental Autoimmune Encephalomyelitis Model of Multiple Sclerosis

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Abstract
Objectives: Based on evidence that pregnant women with multiple sclerosis (MS) show a decline in the relapse rate during the third trimester and an increase during the first 3 months postpartum, the suggestion was made that high levels of circulating sex steroids are responsible for pregnancy-mediated neuroprotection. As both estradiol (E_2) and progesterone exert neuroprotective and myelinating effects on the nervous system, the effects of sex steroids were studied in the experimental autoimmune encephalomyelitis (EAE) model of MS. ***Methods:*** EAE was induced in female C57BL/6 mice by administration of a myelin oligodendrocyte protein ($MOG_{40–45}$) peptide. Clinical signs of EAE, myelin protein expression and neuronal parameters were determined in mice with or without hormonal treatment. ***Results:*** Progesterone given prior to EAE induction attenuated the clinical scores of the disease, slightly delayed disease onset and decreased demyelination foci, according to luxol fast blue staining (LFB), myelin basic protein (MBP) and proteolipid protein (PLP) and mRNA expression. Motoneuron expression of Na,K-ATPase mRNA was also enhanced by progesterone. In turn, combined E_2 plus progesterone therapy more effectively prevented neurological deficits, fully restored LFB staining, MBP and PLP immunoreactivity and avoided inflammatory cell infiltration. On the neuronal side, steroid biotherapy increased brain-derived neurotrophic factor (BDNF) mRNA. ***Conclusion:*** Early treatment with progesterone alone or more evidently in combination with E_2 showed a clinical benefit and produced myelinating and neuroprotective effects in mice with $MOG_{40–45}$-induced EAE. Therefore, sex steroids should be considered as potential novel therapeutic strategies for MS.

Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis

Multiple sclerosis (MS) is an inflammatory, neurodegenerative disease of autoimmune origin that damages the central nervous system (CNS). It is driven by myelin-specific CD4+ T helper 1 (Th1) cells and their products tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and metalloproteinases [1], although an important role for IL-17 is now emerging [2]. MS affects 2.5 million people worldwide and 1 in 1,000 people in western countries,

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showing a 2:1 female to male preference [3, 4]. MS presents several clinical forms: a relapsing/remitting form, primary progressive or secondary progressive forms. Pathological findings in MS include a chronic inflammatory response of the spinal cord and brain which undergo demyelination, axonal loss and neuronal dysfunction, leading to a progressive neurological disability. Current treatments for MS are based on immunomodulatory drugs, which unfortunately are only partially effective [5].

A useful animal model of MS to study etiopathogenic mechanisms and test the efficacy of novel pharmacological treatments is experimental autoimmune encephalomyelitis (EAE). EAE is an immune-inflammatory disease that can be induced in different animal species, such as rats, mice, guinea pigs and rabbits. Whereas most authors consider EAE the best available model for MS, this view is not universally accepted [6]. In spite of the caveats imposed by species differences and inducing factors, EAE has provided substantial information regarding MS. EAE is induced by administration of several myelin proteins, including myelin oligodendrocyte glycoprotein (MOG). MOG is expressed on the outer surface of oligodendrocytes and its administration leads to many features resembling MS. MOG-reactive antibodies have a demyelinating capacity, and when EAE is induced by administration of whole spinal cord homogenates, the disease is partly caused by MOG-specific antibodies [7]. The spinal cord of EAE animals presents low levels of MOG but also of the major central myelin proteins: myelin basic protein (MBP) and proteolipid protein (PLP), besides a concomitant loss of axons and a notorious neuronal dysfunction. Motor deficits ensued in a transitory or permanent fashion [8]. In addition to abnormalities of myelin, axons and neurons, spinal cord pathology of EAE includes infiltration of inflammatory cells, microglial activation, astrocytosis and proliferation of oligodendrocyte precursor cells. Clinically, mice and rats afflicted with EAE show typical neurological deficits, ranging from loss of tail tonicity to several degrees of paralysis and death [8, 9]. There is a strong believe that restoration of myelin sheaths in EAE – a factor of critical importance for MS – will greatly preserve motoneuron function and axonal survival [10].

Basis of Sex Steroid Protection in Neurological Diseases

The disease-modulating effects of estrogens have been demonstrated in CNS injury, ischemia, neurodegeneration and aging. Estrogens qualify as 'neuroprotectants', as shown by many workers in the field [11, 12]. Thus, estrogens prevent cell death, increase neuronal survival and neurite outgrowth, stimulate synaptogenesis and regulate neurotransmission in various experimental situations. In the hippocampus, a recognized estrogen target, estrogens protect against glutamate toxicity, glucose deprivation, FeSO toxicity and amyloid-β peptide toxicity, the hallmark of Alzheimer's disease [reviewed in 13]. Some of the estrogen effects could be genomically mediated, due to interaction of ligand with the estrogen receptor isoforms α or β (ERα, ERβ) produced by different genes. In addition, several types of membrane ER have been found in the CNS, coupled to ion channels or second messengers, pointing out the multiplicity of targets modulated by estrogens in the CNS. The spinal cord is also an estrogen target, with ER principally expressed by neurons in the dorsal horn [14]. Following spinal cord injury in rats, systemic estradiol (E_2) reduces apoptosis of neurons and glial cells and enhances neurological recovery [15]. It has been suggested that neuroprotective effects of E_2 are mediated in part by upregulation of the antiapoptotic gene bcl-2 and also by interaction with growth factors such as IGF-1 (insulin-like growth factor 1) and BDNF (brain-derived neurotrophic factor). Similarly to growth factors, estrogens activate the MAPK (mitogen-activated protein kinase), ERK (extracellular-regulated kinase), PI_3K (phospatidyl-inositol-3-kinase), CREB (cyclic AMP response element-binding protein) and Src pathways [16]. ERα and ERβ are expressed by oligodendrocytes in vivo and in vitro, and these cells are protected from cytotoxicity if E_2 is added to the culture medium [17]. Therefore, in addition to their well-known reproductive function, estrogens are important neuroprotective and myelinating factors if damage, degeneration or aging impact upon the CNS.

A second steroid widely recognized for its function in reproduction and pregnancy is progesterone. In addition, progesterone is now a recognized promyelinating and neuroprotective factor for both central and peripheral nervous system diseases. After experimental brain and spinal cord injury, progesterone promotes motoneuron survival, protects cultured neurons against glutamate toxicity and normalizes defective functional parameters of injured neurons. Given prior to middle cerebral artery occlusion, it reduces infarct size and clinical deficits as reviewed by Schumacher et al. [18]. In a mouse motoneurodegeneration model, progesterone slows the development of the disease, increases survival, axonal transport, the expression of neurotrophic factors and decreases oxidative stress [19]. In conjunction with

neuronal effects, progesterone strongly influences myelin synthesis in the CNS and peripheral nerves. First described following cryolesion of the rat sciatic nerve, the myelinating effects of progesterone have now been shown in organotypic slice culture of 7-day-old rat and mouse cerebellum and in toxin-induced demyelination in old male rats [20, 21]. Following loss of myelin due to injury, progesterone increases the proliferation and differentiation of oligodendrocyte precursor cells (NG_2 cells) that play an important role in remyelination [22]. Genomic effects of progesterone are produced after binding to the intracellular receptors isoforms A and B (PR-A, PR-B), produced by alternate splicing of a single gene. In some CNS regions, PR is induced by estrogen priming whereas in other regions, such as the spinal cord, PR is constitutive. Effects on neurons and glial cells may be due to genomic mechanisms, since PRs are found in motoneurons, oligodendrocytes and Schwann cells [18, 22]. PR intracellular signaling occurs after binding to hormone response elements in DNA followed by transcriptional activation; however, PR may directly interact with Src tyrosine kinases, with activation of the MAPK pathway [23].

It is also known that neuroprotection can be provided by the progesterone metabolite allo-tetrahydroprogesterone (THP) which does not bind to PR. Recent findings of several types of membrane PRs (α, β, γ, 25 DX) support the fact that nongenomic, membrane-associated events participate in neuroprotection and myelination [18]. In this respect, an early report by Majewska et al. [24] has shown that THP modulates the GABA A receptor, an event linked to neuroprotection. Thus, progesterone effects on neuroprotection and myelination involve multiple factors.

Progesterone effects are of great relevance for immune diseases. For instance, increased progesterone levels during pregnancy modulate the immune system, changing a Th1 proinflammatory response into a Th2 anti-inflammatory response [3, 5]. This change may be mediated by PR expressed by lymphocytes and decidual cells, which respond to the hormone by synthesizing a protein known as progesterone-induced blocking factor (PIBF). PIBF plays an important role in immunomodulation [25]. A recent finding has been that galectin-1 (Gal-1), a glycan-binding protein, shows a synergistic effect with progesterone. Supplementation of progesterone in stress-challenged pregnancies in mice increases Gal-1 expression, and it has been suggested that Gal-1 mediates upregulation of PIBF through the action of progesterone [26]. Other evidence supports a role of progesterone in immunomodulation. For instance under the influence of progesterone, activated lymphocytes secrete the noninflammatory cytokines IL-3, IL-4, IL-10 and reduce the inflammatory cytokines IFN-γ, TNF-α and IL-2 [25]. Immunomodulatory effects of progesterone are extended beyond pregnancy. Following traumatic brain injury, progesterone decreases the surge of the inflammatory factors NF-κB p65, complement factor 3, TGFβ2 and IL-1β [27].

Evidence for the Involvement of Steroid Hormones in MS and EAE

It is known that the incidence of MS is twice as frequent in females as opposed to males during reproductive age. Early studies showed an association between lesion size of MS patients, low circulating progesterone levels and high E_2 levels during the sex cycle, suggesting a beneficial role of progesterone [28, 29]. Furthermore, a common observation is that female patients with MS show a decline in the rate of relapses during the third trimester of pregnancy and a significantly greater incidence of relapses within 3 months postpartum [4, 5]. The PRIM study has confirmed that the mean rate of relapses decreases during pregnancy but increases upon delivery [3]. This change is not exclusive of MS, since another autoimmune disorder – rheumatoid arthritis – also improves during pregnancy. The pregnancy effect on MS is believed to be due to altered hormonal profiles, which favor the production of anti-inflammatory cytokines and dump proinflammatory factors [25].

The observation that pregnancy has a positive effect on the prognosis of MS sparked off the analysis of a role of pregnancy hormones in MS. The European multicentric trial POPART-MUS is currently studying postpartum women with MS that receive a mixture of a low potency estrogen – estriol – and a synthetic progestin to avoid the postpartum relapses of MS [4]. This clinical trial represents an important step towards the elucidation of the role of steroid hormones in MS. However, to fully understand the molecular changes taking place in the spinal cord and brain at the time of hormonal treatment(s), experimental models are needed for in vivo and ex vivo determinations.

The beneficial effects of pregnancy observed in women with MS are also observed in animals with induced EAE, such as rats, guinea pigs, mice and rabbits [30, 31]. These observations stimulated the analysis of the effectiveness of different steroid hormones in the EAE model.

As discussed by Offner [32], current studies are based on the possibility that steroids inhibit encephalitogenic T cells and the migration of immune cells into the CNS. Nonetheless, there is increased recognition that steroids also exert direct neuroprotective effects on myelin, axons and neurons, all of which are damaged in EAE.

Regarding this issue, data are available for the therapeutic consequences of the treatment of rodents with EAE employing glucorticoids, androgens, estrogens and progesterone. Glucocorticoids are potent immunosuppressors and can suppress EAE [33], although prolonged treatment leads to secondary undesirable effects. Dehydroepiandrosterone, an adrenal androgen that circulates in amounts equivalent to cortisol in both men and women, can suppress clinical development of EAE in SJL/J mice and reduce Th1-mediated immune responses [34]. Estrogens have been the most explored compounds regarding beneficial effects in EAE animals. For example E_2 offers protection against development of EAE when given prior to disease induction, an effect involving ERα but not ERβ [32]. The involvement of ER isoforms in EAE has been further elucidated using ER-selective ligands. In this case, propylpyrazol triol, an ERα-selective agonist, suppresses EAE, whereas the ERβ agonist Way-202041 lacks effect [1]. Both compounds have been tested prior to disease induction. Instead, the synthetic estrogen ethynyl estradiol and fluasterone (a synthetic androstene derivative with estrogen-like properties) are also effective when given at the onset of EAE. Bebo et al. [35] found that low-dose treatment with E_2 producing diestrous circulating levels of E_2 can significantly reduce clinical manifestations of EAE in male and female mice. Estriol, an estrogen metabolite exhibiting low estrogenic potency, ameliorates EAE in mice provided serum estriol levels similar to those of late pregnancy are achieved [35, 36]. The same authors have not found a positive effect of progesterone.

It has been reported that progesterone has variable effects on EAE, ranging from inactivity, to producing increased vulnerability of neurons during the course of EAE, to disease improvement when given together with E_2 [5, 37, 38]. In particular, the synthetic progestin medroxyprogesterone has shown protective effects in EAE. Interestingly, progesterone enhances estrogen's beneficial effect in mice with collagen-induced arthritis, another immune-related disease [39]. Next, we will sum up our recent findings in EAE mice receiving progesterone monotherapy or subjected to combined therapy with E_2 and progesterone.

Effects of Progesterone on the Clinical Outcome and Spinal Cord Neuropathology of EAE Mice

Based on the premises that progesterone has neuroprotective and promyelinizing effects in the CNS following injury and neurodegeneration, we studied if progesterone protection could be extended to mice with EAE. To this end, adult female C57BL/6 mice were immunized with MOG_{40-54} [40], and 1 week before EAE induction, the animals received single pellets of progesterone weighing either 20 or 100 mg or remained free of steroid treatment. Disease severity was scored as previously published for EAE mice: grade 0 = no signs, grade 1 = partial loss of tail tonicity, grade 2 = loss of tail tonicity and difficulty in righting, grade 3 = unsteady gait and mild paralysis, grade 4 = hind limb paralysis and incontinence, and grade 5 = moribund or death. EAE in hormone-free animals developed approximately on day 10 and mice were sacrificed on day 16 when the disease was still in an acute phase. Serum progestins at the time of sacrifice, measured by radioimmunoassay, were low in hormone-free EAE mice (4.4 ± 2.1 ng/ml), increased 10-fold in animals with the 20-mg progesterone pellet (39.5 ± 7.4 ng/ml, $p < 0.05$ vs. EAE) and further increased 20-fold in mice with the 100-mg progesterone pellet (86.5 ± 10.6 ng/ml, $p < 0.001$ vs. 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 (≈40 ng/ml, unpubl. data).

Mice were monitored daily for weight loss and neurological signs of EAE. All MOG_{40-54}-immunized mice were clinically afflicted with EAE, and the first signs of the disease appeared on days 9–10 following immunization. Mice in the EAE steroid-free group reached on average 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 progesterone doses, respectively. These groups of progesterone-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.

At the spinal cord level, steroid-naïve EAE mice showed inflammatory cell infiltration, partly of cells belonging to a macrophage lineage. Quantitative analysis of cell infiltration showed that 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 progesterone doses ($p < 0.01$ in both cases).

Furthermore, luxol fast blue (LFB) staining for total myelin demonstrated demyelinated foci in circumscribed areas of the spinal cord of untreated EAE mice. Interestingly, the 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 amounted to 8.2% of the total white matter area in EAE mice, whereas in mice receiving both progesterone doses, LFB-negative foci were less frequent. Quantitative analysis of this variable showed that the percentage of demyelination was decreased by half in hormone-treated mice compared to steroid-naïve EAE mice. 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 immunoreactivity of the dorsal and ventral white matter tracts showed negative foci of myelin protein expression in untreated EAE mice compared to control and both groups of progesterone-treated EAE mice. Areas lacking MBP and PLP immunostaining compared to total spinal cord white matter immunoreactive area were 5.2 and 6.0% for MBP and PLP, respectively. These figures were significantly reduced by the implantation of 20- and 100-mg progesterone pellets ($p < 0.05$ in both cases). The enhancement of myelin protein expression by progesterone was similar for MBP and PLP, and was maximally observed in the dorsal and ventral funiculus of the spinal cord.

Myelin recovery following progesterone treatment was also studied at the PLP mRNA level. In this experiment, accumulated grains due to probe hybridized to PLP mRNA were observed over oligodendroglial cells populating the gray matter and the white matter of the spinal cord. Grain density in controls contrasted with fewer grains in untreated EAE mice. However, treatment of EAE mice with the low progesterone dose (20 mg) showed a 1.3-fold increase compared to untreated EAE ($p < 0.01$), suggesting a progesterone effect at the level of gene expression. However, the stimulatory effect of 100-mg progesterone did not reach statistical significance. This was probably due to a mixed response of oligodendrocytes in this group, since cells with high grain density coexisted with others showing low PLP mRNA expression.

From the functional point of view, it is likely that the focal areas of demyelination observed in the ventral and dorsal funiculus lie behind 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. Instead, myelin recovery in the dorsal and ventral funiculus of the spinal cord in progesterone-treated mice was probably involved in the improvement of clinical scores of EAE mice.

Results employing MOG-induced EAE confirmed neuronal involvement due to the fact that the α_3-subunit Na,K-ATPase mRNA was reduced in motoneurons. The enzyme, which is hormone sensitive in the spinal cord, regulates excitability, neurotransmission and nutrient uptake besides its ion transport properties and its deficit is a leading cause of neurodegeneration [41]. Thus, a decrease in the Na,K-ATPase mRNA, as it occurred in untreated EAE mice, could lead to neuropathology and motor impairment, whereas upregulation of Na,K-ATPase mRNA suggested neuronal recovery. Therefore, progesterone administration produced a moderate delay of disease onset, mildly reduced disease severity, and showed beneficial effects regarding the magnitude of the inflammatory response and the occurrence of demyelination in the spinal cord during the acute phase of EAE.

Effects of Combined Treatment with E_2 plus Progesterone on the Clinical Outcome and Spinal Cord Neuropathology of EAE Mice

To test the effect of the combined steroid therapy, C57BL/6 female mice received subcutaneously a 100-mg progesterone pellet plus a 25-mg E_2 pellet. Steroid-naïve mice received cholesterol only 1 week before EAE induction. EAE was induced using $MOG_{40–54}$ and the immunization protocol described above for the progesterone-only experiments. Progesterone + E_2 implantation profoundly modified the clinical scores of EAE mice, since none of the hormone-treated animals showed any clinical signs of EAE; in contrast the steroid-naïve mice developed EAE with a disease onset of an average of ≈10.2 days. The average peak score was 3.75 and the average disease index 171.6. The ratio of the weight of the uterus to the total body weight was 6.67 in EAE hormone-treated mice compared to 3.26 in the untreated mice (EAE vs. EAE + progesterone + E_2, $p < 0.001$) suggesting that progesterone did not completely prevent the uterotrophic effect of E_2.

Quantitative analysis of LFB, MBP and PLP staining demonstrates a significant difference between untreated EAE mice and progesterone + E_2-treated mice. Areas lacking LFB, MBP and PLP staining were, respectively, 10.8, 8.4 and 8.2% of the total spinal cord white matter

staining or immunoreactive area in EAE mice, whereas demyelinating areas were reduced in mice receiving combined steroid therapy to 2.22, 1.02 and 1.18%, respectively (EAE vs. EAE + progesterone + E_2, $p < 0.05$). Inflammatory cell infiltration was studied using hematoxylin-eosin staining and F4/80 immunostaining. The strongest infiltration was observed in the ventral and dorsal funicular regions of the spinal cord, amounting to 6.8% for hematoxylin-eosin and 14% for F4/80. Infiltration of inflammatory cells was almost negligible in EAE mice receiving progesterone plus E_2 treatment. In this study, we also analyzed the expression of BDNF, a factor produced in neurons that could affect oligodendroglia in a paracrine way [42]. The number of silver grains/μm^2 clustered over the neurons was increased in hormone-treated EAE mice compared to non-treated EAE animals. Quantitative grain counting revealed that progesterone + E_2 treatment increased the BDNF mRNA levels significantly. The average of grain density in EAE mice was 0.2222 $\pm$ 0.009/μm^2 and increased in EAE hormone-treated animals to 0.3144 $\pm$ 0.002/μm^2 (EAE vs. EAE + progesterone + E_2, $p < 0.0001$).

Therefore, in EAE mice treatment with progesterone plus E_2 most effectively prevented neurochemical and neurological changes of EAE mice. Comparable results have been observed in collagen-induced arthritis, where a combined treatment with both progesterone and E_2 induced a more pronounced suppression of collagen-induced arthritis than the suppression induced with E_2 treatment [39]. One further aspect is the recovery of BDNF gene expression by combined steroid therapy. Administration of BDNF can rescue injured or degenerating neurons and induce axonal outgrowth. BDNF had beneficial effects on neurodegenerative diseases [43]. One possible hypothesis is that progesterone and E_2 enhancement of endogenous neuronal BDNF could provide a trophic environment within the inflamed spinal cord and might be part of steroid-activated pathways to provide neuroprotection. Detection of intracellular PR in the spinal cord suggests a role for the classical PR in BDNF expression, whereas an estrogen response element is present in the BDNF promoter which probably drives a positive regulation of BDNF transcription. Therefore, BDNF expression in context could be a compensatory mechanism to repair/recover damaged neurons and oligodendrocytes. BDNF receptors are found in oligodendrocytes and in these cells, BDNF increases myelination [44]. In summary, combined steroid therapy seems to provide almost full protection against development of EAE.

Concluding Remarks

It is now apparent that sex steroid hormones such as estrogens and progesterone have favorable effects on mice with EAE. In our own studies, progesterone treatment attenuated neuropathology and clinical signs of EAE, whereas a combination of high-dose E_2 plus progesterone completely prevented development of the disease and its accompanying neurochemical abnormalities of the spinal cord. These beneficial effects may be produced by a combination of a strong immunosuppression at the systemic level plus the prevention of demyelination and neuroprotection. The latter possibility is supported by reports showing that estrogens and progesterone target neurons and oligodendrocytes, changing the expression of genes involved in neuronal function and myelin formation. It would also be crucial to elucidate if steroid therapy stimulates the proliferation and differentiation of oligodendrocyte precursor cells. These cells are responsible for remyelination of demyelinated regions, an event that is poorly sustained in MS [45, 46]. Sex steroid hormones modulate the oligodendrocyte lineage in culture, and they increase the proliferation of oligodendrocyte precursor cells whether demyelination is induced by injury, administration of neurotoxins or whether it spontaneously originates in the course of aging. In the peripheral nervous system, progesterone and derivatives increase myelin gene transcription and induce remyelination after nerve injury and in diabetes-induced peripheral nerve degeneration [18, 47]. A similar effect takes place in the injured spinal cord when animals receive progesterone or estrogens [15, 22]. The advantage of using estrogens plus progesterone bitherapy is that this regimen resembles the hormonal status during pregnancy, in which relapses become attenuated or disappear. Although present efforts towards diminished inflammation and CNS destruction in MS patients relied on immunomodulatory drugs, evidence that sex steroids promote remyelination and neuroprotection should encourage novel approaches to restoration of myelin synthesis and neuronal function for neurodegenerative diseases including MS.

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