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MANUSCRIPT TO BE CONSIDERED FOR THE "STEROIDS AND NERVOUS SYSTEM " SPECIAL ISSUE

NEUROSTEROIDOGENESIS AND PROGESTERONE ANTI-INFLAMMATORY/

NEUROPROTECTIVE EFFECTS

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Short title : Progesterone and neurosteroidogenesis.

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ABSTRACT

Progesterone shows anti-inflammatory and promyelinating effects in mice with autoimmune experimental encephalomyelitis (EAE), a commonly used model for multiple sclerosis (MS). Since neurosteroids have been implicated as protective factors for MS and EAE, we analyzed the expression of neurosteroidogenic enzymes in the compromised spinal cord of EAE mice. EAE was induced in female C57Bl6 mice and killed on day 16 after induction. Progesterone was given by pellet implantation 1 week before EAE induction. Untreated EAE mice showed decreased mRNAs for the steroidogenic acute regulatory protein (Star), voltage-dependent anion channel (VDAC), cholesterol side-chain cleavage (P450scc) , 5α-reductase, 3α-hydroxysteroid dehydrogenase (3α-HSOR) and aromatase, whereas changes of 3β-hydroxysteroid dehydrogenase (3β-HSD) were not significant. mRNA of 18 Kd translocator protein (TSPO) was elevated, concomitantly with a reactive microgliosis. EAE mice also showed abnormal mitochondrial ultrastructure in axons and neuronal bodies, and reduced expression of fission and fusion protein mRNAs. Progesterone pretreatment before EAE induction increased Star,VDAC, P450scc, 5α-reductase type I, 3α-HSOR and aromatase mRNAs and did not modify 3β-HSD. TSPO mRNA was decreased, possibly due to reversal of microgliosis. Progesterone pretreatment also improved mitochondrial ultrastructure and increased fission/fusion protein mRNAs. These mitochondrial effects may be part of progesterone recovery of neurosteroidogenesis. The enzymes 3β-HSD, 3α-HSOR and 5α-reductase are also responsible for the formation of androgens. Since MS patients and EAE rodents show changes of central androgen levels, it is likely that together with progestins and estrogens, neuroandrogens afford neuroprotection for EAE and MS. Data reviewed in this paper suggest that enhanced synthesis of neurosteroids contributes in an auto/paracrine manner to reinforce the neuroprotective and anti-inflammatory effects of exogenous progesterone given to EAE mice.

A ROLE OF STEROIDS AND NEUROSTEROIDS IN AUTOIMMUNE EXPERIMENTAL ENCEPHALOMYELITIS AND MULTIPLE SCLEROSIS

Multiple sclerosis is a disease of autoimmune origin, characterized by demyelination, faulty oligodendrocyte function and neurodegeneration [1, 2]. MS affects mostly females, with a female to male ratio of 2.3 [3]. In addition to gender differences, participation of sex steroids in MS is indicated by the fact that pregnant women with MS show a decline in relapses during the third trimester of pregnancy, at the time when progesterone levels become highly elevated, whereas relapses resume after progesterone levels decrease following parturition [4]. These clinical findings support an anti-inflammatory role of progesterone for MS, in similarity to the antagonism of the immune response at the maternal-fetal interface during pregnancy. Notoriously, MS patients show decreased levels of allopregnanolone, in agreement with decreased gene expression of the 3α-HSOR enzyme [5], while dehydroepiandrosterone (DHEA) levels have not been related to altered expression of any enzyme. Moreover, MS patients also present increased levels of progesterone and its reduced metabolites in cerebrospinal fluid and plasma, and suppressed levels of several neurosteroids, including allopregnanolone in the white matter [6] .These findings support that in MS patients, changes of steroids and neurosteroids may associate with disease outcome.

In similarity with clinical findings, the anti-inflammatory effects of progesterone and reduced metabolites have been substantiated in experimental models of MS, including autoimmune experimental encephalomyelitis (EAE), cuprizone intoxication and lysolecithin demyelination of the spinal cord [7-11]. Regarding the EAE model, we have shown that progesterone pretreatment a week before disease induction reduces cell infiltration, increases myelin basic protein (MBP) and proteolipid protein (PLP), decreases microgliosis and astrogliosis, prevents axonal loss, decreases the proinflammatory mediators TLR4, TNFα, its cognate receptor TNFR1, prevents neuronal dysfunction and enhances motor behaviour [7-9]. In our experience, progesterone given at the time of EAE induction or in animals with established disease is inactive, supporting a protective role for this steroid. However, other reports have shown that progesterone treatment at the time of EAE induction reduces clinical scores, decreases proinflammatory and increases

anti-inflammatory chemokines [10]. Another group has shown that progesterone treatment of clinically affected mice induces nuclear sublocalization of the Olig1 transcription factor followed by remyelination and clinical benefit [11]. The effectiveness of progesterone for chronic EAE has been shown by Gatti et al [12]. These authors have administered progesterone to Dark Agouti rats every other day from the day of EAE induction for 21 days, and killed the animals on day 45 post-induction. This treatment protocol decreases microglia activation, and increases Na,K-ATPase and MPB, indicating steroid effectiveness for chronic EAE [12]. In addition to EAE, progesterone is effective in cuprizone-induced demyelination. In cuprizone-intoxicated mice, progesterone given for 2 weeks alleviates demyelination, decreases apoptosis and increases markers of oligodendrogenesis [13]. In the study of El-Etr et al. [14] , progesterone or the synthetic progestin Nestorone delivered s.c. from implanted minipums increases myelin formation in corpus callosum and cerebral cortex, the number of mature oligodendrocytes and their progenitors. Therefore, these last studies support that progesterone also plays a therapeutic role in rodents with EAE and in cuprizone-induced demyelination.

In addition to the natural hormone, preclinical trials for EAE have also used the progesterone metabolite allopregnanolone (tetrahydroprogesterone). Daily injections of allopregnanolone to immunized mice from the onset of clinical symptoms until the peak of the disease (days 16-17) reduces neuroinflammation, myelin and axonal injury and enhances motor behavior [5].

Moreover, the neuroprotective and anti-inflammatory effects of progesterone is not confined to MS models, because it also occurs in Wobbler neurodegeneration, traumatic brain injury (TBI) and spinal cord transection [15 – 17]. The reported favourable effects of progesterone, its reduced metabolites or synthetic progestins in EAE have been considered direct effects of the exogenously given compounds on CNS target molecules. However, the neuroprotective, promyelinating an anti-inflammatory properties of progestins are also shared by steroids locally synthesized in the nervous system (neurosteroids). Along this venue, we have hypothesized that changes of locally synthesized steroids may potentiate the beneficial effects of the peripherally administered compounds. This review summarizes work performed in our laboratories regarding changes of neurosteroidogenesis in EAE mice and the response of neurosteroid pathways to progesterone.

PROGESTERONE AND EXPRESSION OF NEUROSTEROIDOGENIC ENZYMES IN EAE MICE

Neurosteroidogenesis, i.e., the synthesis de novo of steroids by the nervous system, is a dynamic process subject to modulation by a considerable number of factors. Fluctuations of neurosteroidogenesis occur under physiological conditions such as brain development, ovarian cycle and pregnancy and pathological conditions such as neuropsychiatric disorders, Alzheimer´disease, Parkinson´s disease, MS, Nieman-Pick type C disease, diabetic neuropathy, peripheral neuropathy, traumatic brain injury, spinal cord injury and stroke (for comprehensive reviews see [18 - 20].

Neurosteroidogenesis involves translocator proteins and enzymes responsible for the formation of progesterone and reduced derivatives, androgens and estrogens. Baulieu first described that the brain content of certain steroids remained after ablation of peripheral endocrine glands, and that their brain concentration in some cases outnumbered their blood concentration [21]. The term "neurosteroid" was coined to include the synthesis of steroids by neurons, astrocytes, oligodendrocytes and Schwann cells pertaining to the central and peripheral nervous system. These cells contained the required machinery for steroid synthesis, starting with the mitochondrial transduceosome. The transduceosome complex of the CNS is similar to the mitochondrial complex described for peripheral endocrine glands, such as adrenal, ovary, testes and placenta. According to Papadopoulos and Miller [22]] neurosteroidogenesis starts by activation of the Star protein that brings access of extra-mitochondrial cholesterol to the mitochondria. Once brought into the mitochondria, cholesterol is transported via the channel proteins 18 Kd translocator protein (TSPO), the voltage-dependent anion channel (VDAC) and accessory proteins including TSPO-associated protein 7 (PAP7, ACBD3 for acyl-CoA-binding-domain 3), and protein kinase A regulatory subunit 1α (PKAR1). Inside the mitochondria, the cholesterol side chain clavage enzyme (P450scc) splits the side chain of cholesterol converting it into pregnenolone. Figure 1 shows a schematic view of the transduceosome and enzymes involved in the synthesis of progesterone and its metabolites in the mitochondria and endoplasmic reticulum.

In the following steps, the conversión of pregnenolone into progesterone leads to the synthesis of androgens and estrogens, which require the enzymes of the endoplasmic reticulum (ER) of several cell types. For example, metabolism of

progesterone into dihydroprogesterone (DHP) by 5α-reductase is present in oligodendrocytes and astrocytes [23, 24], whereas 3α-hydroxysteroidoxireductase (3α-HSOR) is present in oligodendrocytes, astrocytes and neurons of the spinal cord [25] and metabolizes DHP into the potent GABA(A) receptor agonist allopregnanolone [26, 27] (Figure 1). Furthermore, progesterone is also metabolized into androgens and estrogens. The latter enzyme, aromatase is normally detected in the endoplasmic reticulum of neurons, but becomes highly expressed in astrocytes after brain injury or in Alzheimer`s disease [28].

Here, we report the changes of gene expression of Star, other mitochondrial components of the transduceosome complex and P450scc in the spinal cord of control and EAE mice with or without progesterone pretreatment. The experimental design for EAE induction involved female C57Bl6 mice, injected with a MOG$_{40-54}$ peptide dissolved in artificial cerebrospinal fluid, pertussis toxin and mycobacterium tuberculosis to boost the immune system [7] .For steroid treatment, a single progesterone pellet (100 mg) was implanted into the neck of mice a week in advance of EAE induction. This method resulted in plasma levels of progesterone similar to those of pregnant mice. Animals were killed on day 16 after induction and the spinal cord dissected out. RNA was extracted from the spinal cord, retro-transcribed into DNA and subjected to amplification using sequence specific forward and revers primers to amplify the expression of the required genes. Full details are given in Garay et al [7]. As shown in Figure 2, untreated EAE mice showed a pronounced decrease of Star, VDAC and P455scc mRNA compared to the spinal cord of control mice. Progesterone pre-treatment of EAE mice recovered Star, VDAC and P450scc mRNA to control levels, which were significantly higher than those of steroid-naive EAE mice. Instead, TSPO showed an opposite profile, with a high increase in untreated EAE mice and a decrease in those mice receiving progesterone. In order to understand the discrepancy in the expression of the transduceosome components, it should be recalled that TSPO is also produced by activated microglia. Therefore, targeting TSPO with progesterone likely produced a beneficial effect, because reactive microglia is the source of proinflammatory cytokines and enzymes, which cause neuronal and oligodendrocyte damage during neuroinflammation [29 - 31]. Supporting these studies, we have previously described up-regulation of microglia and inflammatory markers in untreated EAE mice, which decreases when

mice receive progesterone or the synthetic progestin Nestorone [9, 32]. However, although a reduction in TSPO was evident in EAE mice pretreated with progesterone, it remained significantly higher than control mice (p<0.01). This positive change in TSPO levels of progesterone-treated EAE mice suggests that progesterone might act by ameliorating enzymes and proteins related to neurosteroidogenesis independently from effects on the inflammatory response. As a matter of fact, a role of TSPO in anxiety disorders, peripheral nerve injuries and axonal neuropathies indicates a strong effect of this protein on neurosteroid formation [19, 20, 22, 39].

Therefore, regarding mitochondrial neurosteroidogenesis, induction of EAE impairs mitochondrial neurosteroid enzymes expression, whereas progesterone pretreatment shows a protective effect on these metabolic pathways. It is no unreasonably to conclude that mitochondria of several neurosteroidogenic cells participate in these events. Regarding Star, it has been described in neurons and astrocytes [33- 34]. Our recent confocal laser microscopy observations have shown colocalization of Star immunoreactive protein with CC1+ oligodendrocytes, glial fibrillary acidic protein+ astrocytes, as well as the presence of immunoreactive Star in motoneurons (Garay et al., unpublished results). Reduced levels of transduceosome components indicate that EAE may damage the cell types and organelles expressing these molecules. In the case of VDAC, it is found predominantly in neuronal mitochondria [30], whereas P450scc is a mitochondrial-based enzyme found in neurons, astrocytes and oligodendrocytes [35, 36]. These data suggest that most spinal cord cells damaged during EAE become protected by progesterone treatment. This protective effect would be possible because neurons, oligodendrocytes and astrocytes express intracellular progesterone receptors (PR), membrane receptors (mPR) and other binding molecules [16, 35] which singly or in a combinatory fashion afford neuroprotection.

Since the first enzyme and transport proteins responsible for neurosteroidogenesis are found in the mitochondria, opposite changes of this organelle are expected to occur in EAE and in progesterone-treated EAE mice. The presence of aberrant mitochondria has already been shown in EAE mice and MS patients [38, 39]. Electron microscopy (EM) of spinal cord motoneurons from EAE mice demonstrated that proximal mitochondria in the neuronal body as well as distal

mitochondria in the axon were heavily vacuolated with lack of cristae. Additionally, the axons of EAE mice were surrounded by a disrupted myelin sheath. In contrast, both proximal and distal mitochondria from progesterone-treated EAE mice showed a better conserved ultrastructure, with reconstituted myelin sheath in the axons. Thus, morphology studies support the protective effects of progesterone on mitochondria ultrastructure and also suggest effects on axonal remyelination. Furthermore, mitochondrial dynamics (i.e. fission and fusion transition states), a process important for mitochondrial efficiency, was also faulty in EAE mice. This was shown by a reduction of the Fis1 (fission1) and Mfn2 (mitofusin 2) mRNAs in EAE mice obtained by qPCR.Progesterone pretreatment avoided these alterations and re-established Fis1 and Mfn2 mRNAs to control levels [40]. Therefore, changes in morphology and in mitochondrial dynamic associate with an enhanced neurosteroidogenesis in the progesterone-treated EAE mice.

Our studies have also shown that neurosteroidogenesis of EAE mice was abnormal in the endoplasmic reticulum (ER) . In this structure, (Figure 3), we found low expression of 5α-reductase and 3α-HSOR mRNA, suggesting impairment of progesterone metabolism into its reduced derivatives DHP and allopregnanolone. These findings receive support from previous publications showing reduced levels of DHP in EAE rats and low levels of allopregnanolone in the brain of MS patients and EAE mice [5,12]. Regarding the cellular synthesis of DHP and allopregnanolone, Patte-Mensah et al. [25] demonstrated that oligodendrocytes and neurons can synthesize these neurosteroids, suggesting that these cell types are damaged during EAE and become protected following progesterone treatment. Finally, changes of aromatase expression indicate that EAE spinal cord may be estrogen depleted, whereas progesterone recovers the aromatization of androgens into estrogens. Estrogens have convincingly been shown to ameliorate EAE neuropathology. According to some authors this process is mediated via ERα signalling in astrocytes, considered the producers of proinflamatory mediators [41]. Instead, others have postulated that an ERβ agonist present in oligodendrocytes causes remyelination and has important implications for human use [42].

Some of the enzymes mentioned for the ER (i.e. 3β-HSD, 3α-HSOR and 5α-reductase) are also responsible for the formation of androgens. Since MS patients and EAE rodents show changes of central androgen levels, it is likely that neuroandrogens also afford neuroprotection for EAE and MS [43].

MECHANISMS OF ACTION OF PROGESTERONE

There is general consensus that progesterone shows several effects in the CNS unrelated to female reproductive behaviour, neuroendocrine regulation or pregnancy These effects concern neuroprotection, myelin formation, anti-inflammation, neurogenesis, stroke and trauma prevention [44]. We propose that neurosteroidogenesis may be considered another target of progesterone. In principle, several molecules and signaling pathways may give rise to these progesterone effects on the pathological spinal cord of EAE mice. It is known that the mechanism of action of progesterone is multifactorial, involving the classical intracellular progesterone receptor (PR), several types of membrane receptors (mPRs), sigma1 opioid receptors, the progesterone receptor membrane component 1 (PRMC1) and GABA(A) [45]. Regarding effects on neurosteroidogenesis, some clues are provided by database computer programs and experimental observations. In the first case, the Alibaba 2.1 transcription factor binding prediction program (http://www.gene-regulation.com/pub/programs/alibaba2/index.html) and the Eukaryotic Promoter Database (http://epd.vital-it.ch/), confirm that the promoter sequence of P450scc, TSPO, 3β-HSD and aromatase contain a PR binding site (PRE), suggesting an enhanced transcriptional effects on these genes. The possible PR binding site on the TSPO promoter sequence suggests a transcriptional effect. However, as reported in the literature and in the present work, progesterone was able to partially decrease TSPO expression, possibly due to an anti-inflammatory action. This effect may resemble progesterone effects in the pregnant myometrium, in which decreased pro-inflammatory gene expression occurs when the PR-A to PR-B ratio favored the PR-B isoform [46]. However, as mentioned before, TSPO levels of progesterone-pretreated EAE mice remained higher than in control mice, supporting a positive modulation of TSPO unrelated to neuroinflammation .

Our experimental results also priorized the intervention of PR in some progesterone effects, because the PR binding agonist Nestorone induces neuroprotection and anti-inflammatory effects in EAE mice, supporting a genomic mechanism for these effects [30]. Furthermore, the work of Giatti et al [12] shown that progesterone treatment of rodents with chronic EAE increases the spinal cord level of DHP, a ligand of the PR. Instead, binding of PR to the promoters of Star, VDAC, 5α-reductase and 3α-HSOR are not predicted by the mentioned databases, suggesting other mediators for progesterone effects on these genes. As already mentioned, allopregnanolone recovers EAE mice from signs of the disease [5]. Allopregnanolone is a typical agonist of the GABA(A) receptor, implying a gabaergic mechanism for neuroprotection for EAE and MS among other recognized neuronal functions [26]. Therefore, progesterone regulation of neurosteroidogenesis in EAE may employ pleiotropic (genomic as well as membrane signaling) mechanisms, depending on the molecule in question.

Because neurosteroidogenesis is linked to neuroinflammation [5], we assumed that changes of PR in inflammation conditions may give us a clue about its role in neurosteroidogenesis. To resolve this question, we studied mice with deletion of both A and B isoforms of the PR (PRKO mouse), but in contrast to our expectations, both the PRKO and wild-type mice raised on a C57BL6/129SvEv background were refractory to EAE induction or progesterone treatment (Garay et al., unpublished results). In this experiment, before EAE induction, PRKO and wild type mice show equal numbers of mature oligodendrocytes, astrocytes and microglial cells, and similar myelin immunostaining. However, PRKO mice showed significantly lower incidence of EAE than the C57BL/6 mice (i.e., less than grade 1 or loss of tail tonicity), whereas spinal cord neurochemistry remained similar to control C56Bl6 mice and wild type mice. This experiment suggested that the strain 129xB6 background precludes EAE induction and the response to progesterone. Consequently, we employed a different neuroinflammation model to test for progesterone effects in inflammation, caused by an acute traumatic lesion of the spinal cord. Within hours after making a spinal cord lesion, both wild type and PRKO mice develop astrogliosis, microgliosis and high expression of the proinflammatory factors IL1β, TNFα and IL6 [14]. While a single progesterone injection given at the time of the lesion blunted the proinflammatory factors and glial reactivity in wild type

mice, these parameters remained stable in PRKO mice [16]. These results strongly support that progesterone modulation of immune responses and glial reactivity in the lesioned tissue required an intact PR. By extrapolation, we assume that the anti-inflammatory effects of progesterone on EAE mice [9] may be PR dependent. Future experimental designs are needed to unveil this possibility.

Literature reports indicate an increasing list of modulators of neurosteroidogenesis. These molecules include inducers such as estrogens [47], progesterone [48], and the neurotransmitter n-methyl-d-aspartate (NMDA) plus calcium [49]. Proteins able to induce neurosteroidogenesis, such as TSPO and PXR [20] represent possible receptors for ligands of increasing interest for pharmacological studies and therapies. Comprehensive reviews on the beneficial effects of TSPO ligands in neurodegenerative diseases and MS models are found in Porcu et al [20] and Ravikumar et al. [50].

Preclinical studies provide the background for the design of clinical trials, and in this sense, progestins have been tested in patients traumatic brain injury (TBI) and MS. In TBI patients, early phase II clinical trials have shown protective effects of progesterone, but two clinical trials named ProTECT and SyNAPSE involving a large number of subjects has failed to replicate these effects [51, 52]. However, concerns about the heterogeneity of patients, lack of reliable biomarkers, statistical evaluation and dose and length of progesterone administration have recently questioned the conclusions of these Phase III trials [53, 54]. Regarding MS, a trial named Popartmus that used combined therapy with nomegestrol plus estriol has been discontinued due to poor enrolling of patients [53]. Therefore, further studies employing different species besides rodents and humans, modified treatment protocols and careful patient segregation will give more definite answers regarding the usefulness of progesterone and derivatives for CNS diseases.

ACKNOWLEGEMENTS

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Figure 1:　　Neurosteroidogenesis in mitochondria and extra-mitochondrial compartment. The transport of cholesterol from the cytoplasm to the inner mitochondrial membrane involves the translocase 18 kDa (TSPO) and the steroidogenic acute regulatory protein (StAR). Both proteins are part of a multiprotein complex named "transduceosome", including the voltage-dependent anion channel (VDAC). At the level of the inner mitochondrial membrane, cholesterol is converted to pregnenolone by cytochrome P450scc. Pregnenolone is then converted to progesterone by the 3β-hydroxysteroid dehydrogenases (3β-HSD), which are either located inside the mitochondria, forming a complex with P450scc, or in the cytoplasm associated with membranes of the endoplasmic reticulum (ER). Progesterone is irreversibly converted to 5α-dihydroprogesterone by the 5α-reductases. In neural cells, both progesterone and 5α-dihydroprogesterone bind to the intracellular progesterone receptors (PR). 5α-dihydroprogesterone is metabolized to allopregnanolone by the 3α-hydroxysteroid dehydrogenase (3α-HSD). Allopregnanolone does not bind PR but is a positive modulator of $GABA_A$ receptors. $GABA_A$, γ-aminobutyric acid type A; OMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; PRE, progesterone response element.

Figure 2: Expression of the mRNA of steroidogenic acute regulatory protein (Star) and mitochondrial neurosteroidogenic enzymes in the spinal cord of EAE mice without or with progesterone pretreatment. A: mRNA levels of Star were decreased in EAE mice (** $p<0.01$ vs control) and increased in the EAE group receiving progesterone (&& $p<0.01$ vs EAE). B: The mRNA for the 18Kd translocator protein

(TSPO) was increased in EAE mice (*** p<0.001) but decreased if these mice receive progesterone ([&] p<0.05). TSPO mRNA of the EAE receiving progesterone was still higher than those in control mice (^{##} p<0.01). C: mRNA expression of the voltage-dependent anion channel (VDAC) was lower than control in EAE mice (***p<0.001) but increased when these mice received progesterone (^{&&&}p<0.001). D: Expression of the cholesterol-side chain cleavage enzyme (P450scc) was lower in EAE vs. control mice (*p<0.05) and recovered if EAE mice received progesterone (^{&&}p<0.01). Statistical analysis (mean ± S.E.M.; n= 6-7 spinal cords per group) employed ANOVA followed by the Newman-Keuls test. Reprinted with permission from Garay et al. [38].

Figure 3: Expression of the mRNA of microsomal (endoplasmic reticulum) neurosteroidogenic enzymes in the spinal cord of EAE mice without or with progesterone pretreatment. A: mRNA levels of 3β-hydroxysteroid dehydrogenase (3β-HSD) showed a large variation, without significant intergroup changes. The mRNA for the 5α-reductase was decreased in EAE vs. control mice (***p<0.001) whereas progesterone treatment of these mice reverted this effect (^{&&&}p<0.001). C: mRNA expression of 3α-hydroxysteroid dehydrogenase (3α-HSOR) was lower than control in EAE mice (**p<0.01) but increased when these mice received progesterone ([&]p<0.05). D: The mRNA for aromatase mRNA was decreased in EAE compared to control mice (**p<0.01) and increased if these mice received progesterone (^{&&}p<0.01). Statistical analysis (mean ± S.E.M.; n= 6-7 spinal cords per group) employed ANOVA followed by the Newman-Keuls test. Reprinted with permission from Garay et al. [38].

A StAR

B TSPO

C VDAC

D P450scc