PROGESTERONE PROTECTIVE EFFECTS IN NEURODEGENERATION AND NEUROINFLAMMATION.

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ABSTRACT Progesterone is a neuroprotective, promyelinating and antiinflammatory factor for the nervous system. Here we discuss progesterone effects in models of motoneuron degeneration and neuroinflammation. In neurodegeneration of the Wobbler mouse, a subset of spinal cord motoneurons showed increased activity of nitric oxide synthase (NOS), increased intramitochondrial NOS, decreased activity of respiratory chain complexes and decreased activity and protein expression of Mn-superoxide dismutase type 2 (MnSOD2). Clinically, Wobblers suffered several degrees of motor impairment. Progesterone treatment restored the expression of neuronal markers, decreased the activity of NOS and enhanced complex I respiratory activity and MnSOD2. Long-term treatment with progesterone increased muscle strength, biceps weight and survival. Collectively, these data supported that progesterone prevented neurodegeneration. To study progesterone effects in neuroinflammation, we employed mice with experimental autoimmune encephalomyelitis (EAE). EAE mice spinal cord showed increased mRNA levels of the inflammatory mediators tumour necrosis factor α (TNFα) and its receptor TNFR1, the microglial marker CD11b, iNOS and the toll-like receptor 4 (TLR4). Progesterone pretreatment of EAE mice blocked the proinflammatory mediators, decreased Iba1+ microglial cells and attenuated clinical signs of EAE. Therefore, reactive glial cells became targets of progesterone anti-inflammatory effects. These results open the ground for testing the usefulness of neuroactive steroids for neurological disorders.

Key words: progesterone; neuroprotection; Wobbler mouse; anti-inflammatory effects; experimental autoimmune encephalomyelitis.

Progesterone´s protective role in motoneuron degeneration

Experimental and clinical evidence indicate a protective role of progesterone in disorders of the central and peripheral nervous system including traumatic brain and spinal cord injury, neuroinflammation, stroke, ischemia, diabetic neurophathy, neurophatic pain and neurodegeneration [1 – 8]. A human neurodegenerative disease targeting motoneurons of the spinal cord, brain stem and motor cortex is amyotrophic lateral sclerosis (ALS) [9]. ALS patients show a fatal outcome in less than 5 years, which can be retarded for a few months by treatment with the anti-glutamatergic drug riluzole [10]. Therefore, alternative procedures to ameliorate the course of this disease are urgently needed.

There are several animal models of ALS, among them the Wobbler mouse [11, 12]. Wobblers suffer a mutation in the gene coding for VPS_{54} (vacuolar vesicular protein sorting) present in chromosome 11. In common with patients with the sporadic form of ALS, Wobblers motoneurons show a relocation of TDP-43 (nuclear transactive DNA-binding protein) from the nuclear to the cytoplasmic compartment and changes of ubiquitin [13]. This finding make Wobblers potential models for sporadic ALS, a form of the disease accounting for about 85-90 % of all cases [11]. Morphologically, anterior horn motoneurons of Wobbler mice undergo a dramatic perikaryal vacuolar degeneration, accompanied by astrocytosis and microglial activation [11, 12, 14,15]. This type of neuronal death is known as cytoplasmic type II cell death or paraptosis [16]. Participation of oxidative stress in this mechanism is supported by the clinical, biochemical and morphological improvement caused by treatment with antioxidants, antiglutamatergic drugs, steroids, nitric oxide inhibitors and neurotrophic factors [17 - 19]. In addition to vacuolation, we demonstrated in

Wobbler mice motoneurons abnormal expression of several molecules such as nitric oxide synthase (NOS), Na,K-ATPase, brain-derived neurotrophic factor (BNDF), choline-acetyltransferase (ChAT) and growth-associated protein GAP-43, in addition to deficits of retrograde axonal transport [19 - 21].

Progesterone protective effects were first studied in symptomatic Wobbler mice showing full-blown expression of the disease, i.e., ambulatory difficulties, muscle atrophy and forelimb flexion. These mice remained untreated or received a single progesterone pellet (20 mg) under the skin of the neck. After 18 days of treatment, vacuolated motoneurons in the spinal cord of progesterone-treated Wobblers were already reduced 6-fold, with a concomitant reduction of NOS active neurons. Deactivation of nitric oxide producing mechanisms could relieve motoneurons from increased oxidative stress [22]. Although the mechanisms of progesterone action were not studied in Wobbler motoneurons, the presence of substantial amounts of the reduced derivatives 5α-dihydroprogesterone (DHP) and 3α,5α-tetrahydroprogesterone (THP) in plasma and spinal (Guennoun et al., unpublished results) cord suggest that progesterone effects could be mediated by different receptors. In this regard, classical intracellular receptors, membrane receptors (α, β, and γ subtypes), the PMC1 (formerly 25Dx) as well as GABA A receptors, that bind progesterone and/ or its reduced metabolites have been demonstrated in the spinal cord [1, 23 - 26].

In addition to decrease vacuolation of motoneurons, exposure of Wobbler mice to progesterone prevented other neuronal abnormalities. These included the expression of neuronal BDNF mRNA and the subcellular distribution of

BDNF protein. Expression levels of neuronal BDNF mRNA, measured by in situ hybridization, were lower in Wobbler mice compared to control mice. The neurotrophin was up-regulated by progesterone in α-motoneurons which innervate the forelimbs muscles, smaller neurons such as γ-motoneurons that control muscle spindle organs, Renshaw inhibitory cells and interneurons of Wobblers [27]. These responses pointed to an intrinsically complex regulation of BDNF expression in the neuronal network of the ventral horn. *Prima facie*, up-regulation of endogenous BDNF should be helpful to Wobbler mice, because treatment of these animals with exogenous BDNF slows motoneuron degeneration, diminishes axon loss and enhances behavioral parameters related to locomotor activity [28]. In a time-course study, we observed that BDNF mRNA, which was already depleted at the presymptomatic stage of the Wobbler disease, continued to be poorly expressed at the symptomatic and late stages. Progesterone treatment replenished BDNF mRNA progesterone preferentially in the last two stages, when motoneuron degeneration was more severe [29]. However, BDNF protein content analyzed by ELISA in ventral horns or by immunostaining of motoneurons, remained normal in steroid-naïve Wobblers at the established stage but was decreased by progesterone, suggesting that the steroid increased anterograde transport and /or release of neuronal BDNF. Working in an autocrine manner, BDNF bins to neuronal tropomiosine-kinase type B receptors (TrkB) and activate gene transcription employing different signaling cascades [30]. Preliminary data showed that TrkB mRNA was reduced in untreated Wobblers and increased following progesterone treatment. Concomitantly, steroid treatment down-regulated the expression of p75[ntr], a pan neurotrophin receptor type mediating death signals after binding neurotrophins (Gonzalez Deniselle et al. unpublished results). Thus, progesterone could play a dual role, in part by stimulation of endogenous BDNF

and TrkB, and in part by decreasing neurotrophin binding to p75 [ntr.] . BDNF released from motoneurons after progesterone stimulation could bind to the TrkB receptors in nearby neurons in a paracrine manner [30]. A paracrine role for BDNF has been proposed following axotomy, degenerative diseases and ischemia [32 - 34]. Besides neurons, other potential targets of neuronal-secreted BDNF are the oligodendrocytes, in which the neurotrophin increases myelin synthesis [34]. We also showed that progesterone-stimulated BDNF in the Wobbler mouse was concomitant to changes of the oligodendrocytes. Wobbler mice showed a depletion of CC1 immunopositive oligodendrocytes in the white matter of the spinal cord, whereas progesterone treatment enhanced the density of BDNF/CC1 double labeled oligodendrocytes at all stage periods of the disease [29]. Thus, BDNF-mediated progesterone effects could protect neurons from degeneration and also promote myelination acting on the oligodendrocytes.

Progesterone treatment of Wobbler mice also resulted in changes in ChAT, the enzyme responsible for acetylcholine synthesis. Untreated Wobbler mice presented a significant reduction in ChAT-immunoreactive motoneurons, indicating they are dysfunctional neurons that lost the ChAT phenotype before dying [27]. A time-course study revealed that ChaT was reduced at all stages of the Wobbler disease [31]. In agreement with the results in Wobblers, ChAT-immunoreactivity is also diminished in the spinal cord of ALS patients [34]. We found that in the forelimb muscles of Wobbler mice, ChAT activity was reduced by 55.3% compared to control animals, which also agreed with similar findings in other laboratories [36]. Depletion of ChAT-IR in neurons and enzyme activity in nerve terminals are not permanent but plastic events, because progesterone induced a significant increment

in ChAT-positive neuronal density in the spinal cord and increased enzyme activity in the muscle. In this way, cholinergic neurotransmission and muscle contraction could be recovered after progesterone treatment of the Wobbler mouse.

During the course of the Wobbler disease, of critical importance is the production of neurotoxic levels of NO by neurons and astrocytes [20, 37]. Excess levels of NO bind to and alter the function of the mitochondrial respiratory chain [22]. Work from other laboratories have already demonstrated that mitochondria from the spinal cord and motor cortex of Wobbler mice present respiratory chain dysfunction with decreased oxygen consumption, decreased complex I and complex IV activities, decreased state 3 and 4 respiration rates, decreased oxidative phosphorylation and aberrant activation of delta PKC that modulates mitochondrial-induced apoptosis [38, 39]. Ultrastructurally, motoneurons from Wobbler mice show mitochondrial membrane disruption, cristolysis and vacuolation [19]. The abnormal mitochondria are a source of free radicals, superoxide anion and nitric oxide due to the high activity of a mitochondrial nitric oxide synthase (mtNOS) [40]. Considering the significant role played by mitochondria in cell metabolism and function, prevention of mitochondriopathy could result in healthier and functional motoneurons.

The possibility that neurotoxic levels of NO caused vacuolation of motoneurons, prompted the analysis of nNOS content. Data gathered by Western blot showed an increased signal for nNOS in mitochondria (mtNOS) from the cervical, but not lumbar spinal cord, of steroid-naïve Wobblers compared to control and progesterone-receiving Wobblers. In contrast, cytosolic nNOS in the same groups seemed unchanged in the cervical portion and weakly present in the lumbar region,

as illustrated in Fig. 1. Measurement of the NADPH-diaphorase activity of NOS by histochemistry demonstrated several staining profiles in the untreated Wobbler, which may correspond to nitrergic motoneurons. In contrast, NADPH-diaphorase active motoneurons were greatly decreased in progesterone-treated Wobblers (Fig.1). As a further step, the expression for nNOS mRNA was determined in ventral horn motoneurons from Lamina IX of the cervical region of the spinal cord by in situ hybridization. However, no significant differences in grain density were obtained between control, Wobbler and Wobbler plus progesterone groups. Similarly, when the nNOS mRNA content was measured in the cervical spinal cord by real time PCR, differences were not found between control mice, untreated Wobblers and Wobbler plus progesterone groups. Altogether, these results suggest that gene transcription was not involved on the effects of the Wobbler mutation and progesterone treatment on mitochondrial mtNOS [41].

Having demonstrated the presence of increased intramitochondrial nNOS in the cervical spinal cord of Wobbler mice, and its reversal by progesterone treatment, we studied the consequences that changes of nNOS could have on respiratory chain components. Determination of mitochondrial complex I activity showed a significant reduction in the cervical and lumbar regions of the spinal cord of untreated Wobbler mice. Progesterone treatment returned complex I activity to normal in the cervical region, .but not in the lumbar region. Complex II-III activity of Wobbler mitochondria from the cervical region showed significantly reduced activity, but progesterone was unable to recover it. Measurement of complex IV activity (cytochrome oxidase) did not produce significant differences between control, Wobbler and Wobbler plus progesterone groups in the cervical or lumbar spinal cord. In conclusion,

progesterone recovered mitochondrial respiratory complex I activity in the cervical spinal cord, the region preferentially affected by the Wobbler neuropathology [41]. .

The mechanism(s) responsible for the increased level of mtNOS in the Wobbler, and the opposing effect of progesterone effect on this parameter seems an important point deserving elucidation. One possibility could be a decreased axonal transport of nNOS to the axonal terminal. Accumulation of the enzyme in the cytoplasm may facilitate its entry into the mitochondria. Although the mechanisms of nNOS translocation are poorly defined, post-translational modifications of the enzyme such as phosphorylation or acylation have been proposed to control the import of this enzyme into the mitochondria [40]. A role of progesterone in these mechanisms remains to be established, although modulation of nNOS transport into mitochondria seems appealing. Since mtNOS and NO may cause motoneuron vacuolation and mitochondriopathy in the Wobbler, a closer look at the interaction between NO and progesterone may bring into light future therapeutic possibilities for neurodegeneration

Oxidative stress is normally opposed by different cellular protective mechanisms, such as the superoxide dismutases (SOD). Within the mitochondria, MnSOD constitutes a leading defence against superoxide anion damage. This raises the question on whether anti-oxidant mechanisms failing in the Wobbler could be restored by progesterone. To elucidate this issue, we studied the effects of the Wobbler mutation and progesterone treatment on MnSOD activity and immunostaining, and found that enzyme activity was reduced by half in the Wobbler mice compared to control and Wobbler mice receiving progesterone. We also found

that the number of MnSOD immunopositive motoneurons was slightly, albeit significantly reduced, in the Wobblers vs. controls, and in this case, progesterone treatment of Wobbler mice normalized the number of MnSOD stained motoneurons [41]. It was interesting to find out that degenerating motoneurons retain their capability to increase mitochondrial MnSOD in response to a protective stimulus, preventing the cellular damage provoked by reactive oxygen species originated in Wobbler motoneurons.

Changes of neurochemical parameters under the influence of progesterone were accompanied by marked clinical improvements. Thus, long-term progesterone treatment attenuated the ongoing atrophy of forelimb biceps bracchii, increased muscle strength and prolonged survival of Wobbler mice [19]. Progesterone protective effects in the Wobbler mouse encouraged studies in patients with neurodegenerative diseases. In this regard, steps were taken to look for a possible role of progesterone in humans with ALS.

Based on the progesterone protective effects in the Wobbler mouse, we assume that if variations occurred in serum progesterone levels in patients with ALS, they may influence the degree of damage to the central nervous system. In line with this hypothesis, we have searched if an association existed between progesterone levels with prognostic factors and survival of patients with ALS [42]. We observed in a population of ALS patients, that endogenous progesterone serum concentrations were positively correlated with better prognostic factors, like survival and length of time from disease onset to diagnosis, and negatively correlated with age, a worse prognostic factor. Likewise, bulbar onset patients showed lower progesterone levels

in comparison to spinal onset patients, a condition of better disease prognosis. The origin of serum progesterone could be the adrenal gland, because gonadal progesterone decreases with aging. Pattachioli et al [43] have shown increased activity of the HPA axis in ALS patients, suggesting hyperadrenocorticism. However, it has still to be ascertained whether progesterone elevation in ALS responds to a pathological activation of the HPA axis or if it is a mechanism to confront neurodegeneration. We have considered that progesterone levels in ALS patients may possible influence ALS prognosis and survival. To this end, it would be crucial to find out if progesterone levels in the cerebrospinal fluid and in post-mortem tissues are also changed in ALS patient, to better elucidate the contribution of neuroprogesterone. In a second study employing ALS patients and age-matched healthy controls, we analyzed the relationship between circulating steroid levels and respiratory function, taking into account that ALS patients end up with assisted ventilation due to respiratory failure. We found that an elevated progesterone/cortisol ratio in blood levels was associated with better forced vital capacity, a measure of good respiratory function [44].

In a third study performed with post-mortem tissues, the spinal cord of control subjects and ALS patients was processed for determination of progesterone receptors (PR) mRNA by PCR and for PR localization using immunostaining. PR was localized in motoneurons, blood vessels and glial cells of both normal and degenerating human spinal cords. PR mRNA was determined in the cervical and lumbar regions of the spinal cord. Results have shown that in the cervical region, mRNA for PR (A+B isoforms) and PR B isoform were similar in controls and ALS. In the lumbar region, ALS patients showed a significantly higher expression of PR B than control subjects. These data

indicated that (a) PR was expressed in the human spinal cord, and (b) the possibility existed for a higher expression of PR in ALS patients. Combined with progesterone levels found in ALS patients, PR data implies that up-regulation of protective factors constitute a reparative effort to attenuate neurodegeneration (Gargiulo-Monachelli et al. unpublished data). Altogether, data obtained in humans suggests the possibility that progesterone treatment may be clinically useful in a chronic neurodegenerative disorder like ALS.

Progesterone effects in a neuroinflammation model

Multiple sclerosis (MS) is an inflammatory disease targeting the central nervous system. MS is considered of autoimmune origin, driven by myelin specific CD4+ T helper-1 (Th1) cells and inflammatory cytokines [45, 46]. There is also strong evidence for neurodegeneration in MS [47]. Intrinsic defects of the oligodendrocytes, with release of myelin proteins and debris that provoke a secondary reaction of the immune system have also been implicated [48]..

In approximately 80% of the cases MS shows a relapsing-remitting course, with a significant decline in the rate of relapses during the third trimester of pregnancy, and a significant return post partum. The absence of MS relapses during pregnancy is believed to be due to the protective and anti-inflammatory effects of sex steroid hormones circulating in high levels in pregnant women, whereas post-partum relapses may be due to decreased levels of circulating steroids [49]. Along the steroid hypothesis, a recent report has shown decreased levels of brain neurosteroids in patients with MS and mice with experimental autoimmune encephalomyelitis (EAE), the most common model of MS [50]. Therefore, the

potential therapeutic benefit of sex steroid hormones for MS patients has been taken into consideration [51].

Commonly, EAE is induced in rodents by immunization with one of the central myelin proteins. Neuropathology of EAE spinal cord includes inflammatory cell infiltration, demyelination with oligodendrocyte loss, microglial activation, axonal loss, astrocytosis and neuronal dysfunction [52 , 53]. Clinical correlates of spinal cord pathology include loss of tail tonicity, rear limb paralysis and even death [53]. Among other treatments, steroids have been successfully employed to prevent the development or halt the progression of EAE [53 - 59]. Therefore, EAE provides a useful model to analyze the effects of progesterone on neuroinflammation.

Thus, based on current demonstrations that the spinal cord is sensitive to the protective effects of progesterone, as exemplified above for the neurodegeneration of the Wobbler mouse, we studied whether progesterone influences the inflammatory reaction of the spinal cord of EAE mice. To this purpose, female C57BL/6 mice were immunized with a myelin oligodendrocyte glycoprotein peptide (MOG_{40-54}). One week before EAE induction, mice received single pellets of progesterone weighing 100 mg or remained free of steroid treatment. On average, mice developed clinical signs of EAE 12 - 13 days following MOG administration. The spinal cord white matter of EAE mice showed circumscribed demyelinating areas, demonstrated by reductions of luxol fast blue (LFB) staining, myelin basic protein (MBP) and proteolipid protein (PLP) immunoreactivity (IR) and PLP mRNA expression [58,59]. In motoneurons, mice with EAE showed reduced expression of neuronal markers and axonal loss. These abnormalities were accompanied by a strong reaction of the cells composing the innate immune system, determined 17 days after immunization.

Thus, strong microglia reactivity, assessed by the microglial markers Iba1 and CD11b occurred in EAE mice. There was also a strong expression of several proinflammatory molecules, including tumor necrosis factor alpha (TNFα) and its receptor type 1, the toll-like receptor 4 (TLR4) and the inducible form of nitric oxide synthase (iNOS), as illustrated in Fig. 2. Notably, there was a 27-fold increase in TNFα mRNA increase in EAE mice [58]. Using double immunfluorescence staining and confocal microscopy, we localized TNFα with markers of microglia (OX42) and astrocytes (GFAP), which confirmed the participation of these cell types in the immune reaction. These inflammatory factors are deeply involved in spinal cord pathology [60]. TLR4 is highly expressed on microglia and other immune cells after trauma and inflammation of the central nervous system [61]. Stimulation of this receptor can lead to activation of NFkB and mitogen-activated protein (MAP) kinases and production of inflammatory mediators including TNFα, IL-6, IL1-b, intercellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1) [62, 63]. In addition, deregulation of TLR4 could be partially responsible for EAE pathogenesis. Studies in vivo and in vitro have revealed that LPS exposure causes TLR4-dependent microglia activation associated with oligodendrocyte death, demyelination, neuronal and axonal loss, i.e., events that typically characterize EAE [63]. TNFα is a target gene downstream of TLR4 activation, explaining its higher expression in the spinal cord of EAE mice. In this regard, high levels of TNFα were identified in acute and chronic MS lesions suggesting its involvement in lesion formation [63]. In EAE, inhibition of TNFα with an IgG1 monoclonal antibody effectively prevented the disease [64].

In our experiments, we found that pretreatment of EAE mice with progesterone diminished microglial cell reactivity, leading to lower immunoreaction for Iba1 and mRNA for the CD11b microglial marker. As shown in Fig. 2, progesterone pretreatment decreased the expression of the mRNAs of the receptor TLR4, of the proinflammatory cytokine TNFα and its receptor TNFR1 and induced a slight reduction of iNOS mRNA in the spinal cord. Instead, other inflammatory molecules up-regulated by EAE were steroid-insensitive. The anti-inflammatory effects of progesterone may be ascribed to direct effects on reactive astrocytes and microglia [58,59]. In addition, progesterone significantly enhanced the expression of transcription factors involved in specification of the oligodendrocyte lineage and myelin repair, increased mature oligodendrocyte density and elevated the expression of mRNAs of the myelin protein MBP and PLP. Clinically, animals receiving hormone treatment\ showed an attenuation of neurological disability, as EAE mice without treatment reached grades 2 – 3 (i.e., monoplegia or paraplegia) whereas those receiving progesterone remained on the average on grade 1 (i.e., loss of tail tonicity) [59].

The immunomodulatory effects of progesterone have been recognized in other situations. Progesterone favors the development of T helper cells producing Th2-type cytokines [65]. Pregnant women with MS show a decrease in the relapse rate during the third trimester ascribed to high levels of estrogens and progesterone [49, 51]. A significant attenuation of the microglial reactivity is observed in progesterone-treated EAE-treated mice probably by a direct steroid action upon these cells. Although the presence of progesterone receptors in microglia is controversial, direct antiinflammatory effects of progesterone and its derivatives on a

microglial cell line have been demonstrated [66]. Thus, impairment of microgliosis may be beneficial to EAE mice because these cells represent an important source of damaging inflammatory mediators. Thus, the decreased expression of TLR4 by progesterone may prevent the activation of proinflammatory genes, demyelination and oligodendrocyte death in EAE. This effect is in accordance with previous in vitro studies showing that pretreatment with pregnant levels of progesterone inhibited the up-regulation of TLR4 expression on murine macrophages exposed to LPS [67]. In traumatic brain injury, up-regulation of TLR4 mRNA is markedly inhibited by progesterone administration [68]. Progesterone attenuation of the high expression of TNFα mRNA in the spinal cord of EAE mice may lead to protection of myelinating cells. Oligodendrocytes and their progenitors are particularly vulnerable to the effects of this cytokine in vivo and in vitro. Indeed, treatment with TNFα-promotes apoptosis of mature oligodendrocytes and inhibits maturation and differentiation of their progenitors (OPC) [69, 70]. Colocalization studies showed that this cytokine was mainly produced by microglia and astrocytes. TNFα interaction with the TNFR1 could be responsible for the cytolytic activity of TNFα [71]. In this way, progesterone anti-inflammatory actions were probably mediated in part by reduction of microgliosis and the mentioned proinflammatory mediators. iNOS expression was also attenuated by progesterone, which confirms results in other systems. Thus, in a spinal cord neurodegeneration model, progesterone decreases nitric oxide production, possibly acting on the nNOS isoform [41]. Further studies are necessary to establish the role of iNOS and the other NOS isoforms in progesterone effects in a neuroinflammatory milieu.

In addition to the inflammatory mediators reported in the present study, other cytokines have been already demonstrated to be under the control of progesterone. These include a decrease of the inflammatory interleukins IL-2 and IL-17 and an increase of the anti-inflammatory IL-10 in EAE mice, decreases of IL-1β and TNFα in brain-injured rats and decreases of IL-1β, transforming growth factor (TGF)b2 mRNAs after brain ischemia [72, 73]. Thus, suppression of the immune response seems a generalized property of progesterone in different experimental conditions.

Therefore, exogenously administered progesterone constituted a novel therapeutic strategy during the acute phase of EAE [14]. However, endogenously produced steroids (neurosteroids) may also play a significant role during the course of EAE. Work of the Melcangi group [74] has shown that in rats with induced EAE, there are dimorphic changes in the levels of progesterone and derivatives in different central nervous regions, as assessed by a highly specific gas chromatography/ mass spectrometry procedure. The authors have concluded that these studies may help to design therapies and possibly sex-specific therapies for MS.

So far, we have observed that progesterone treatment in vivo produced anti-inflammatory effects in EAE mice. In order to elucidate if these effects are entirely due to suppression of the peripheral immune system or to additional local spinal cord effects, we have resorted to a model of primary injury induced by the intraspinal injection of lysophospatidylcholine (LPC) [75]. In this study, C57Bl6 adult male mice remained steroid-naive or received a single 100 mg progesterone implant, which increased circulating steroid levels to those of mouse pregnancy. Seven days afterwards mice receivde a single injection of 1% LPC into the dorsal funiculus of the

spinal cord. This procedure induced local inflammation and demyelination. We observed that progesterone pretreatment of LPC-injected mice increased myelination, stimulated the proliferation of oligodendrocyte precursors and mature oligodendrocytes, and most importantly, attenuated the microglial/macrophage response. Thus, the LPC model supports direct progesterone anti-inflammatory effects in the spinal cord.

It is an accepted fact that progesterone-induced immunosuppression during human pregnancy may prevent relapses of MS. Based on this observation, 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. This clinical trial is supported by demonstrations that estrogens and progesterone protected and /or attenuate EAE development [53 - 59]. The outcome of the POPART-MUS trial is eagerly waited because it may encourage the use of sex steroid hormones as protective factors for human MS.

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Fig.1 : Western blot analysis of nNOS protein in mitochondria and cytosol fraction from control mice, Wobbler mice and Wobbler mice receiving progesterone. (a) Increased signal for nNOS in the cervical, but not the lumbar region of the spinal cord, from Wobbler mice (Wr) compared to control mice (CTL). Progesterone reduced nNOS signal intensity in Wobbler cervical cord (Wr PROG). (b) : Unchanged cytosolic nNOS signal in the cervical and lumbar regions of Wobbler compared to control or progesterone-treated Wobbler. (c) : Densitometric measurement (expressed in arbitrary units, AU) showed significantly higher nNOS protein content in Wobbler mitochondria from the cervical cord (* $p<0.05$) compared to control or progesterone-treated Wobbler. (d): Densitometric analysis of cytosolic nNOS showed similar levels in the cervical or lumbar regions in the three groups studied. (e): Stronger NADPH-diaphorase staining shown by Wobbler spinal cord (middle image) compared to control mouse or progesterone-treated Wobbler (upper and lower images, respectively). Arrowhead in the middle image points to a motoneuron, asterisk to probable endothelial cells. Microphotographs were taken from the cervical region of the spinal cord. Inside bar: 100 μm. Data derived from Gonzalez Deniselle et al. [41].

Fig. 2: Quantitative analysis of the effects of progesterone on proinflammatory mediators in the spinal cord determined by real time PCR. The graphs represent CD11b (**A**), TLR4 (**B**), iNOS (**C**), TNFα (**D**) and TNFR1 (**E**) mRNAs. Statistical comparison showed that EAE up-regulated the microglial marker CD11b, proinflammatory molecules and iNOS mRNAs compared to control mice (CTRL): * $p<0.05$ for TNFα, CD11b, and TLR4, ** $p<0.01$ for TNFR1 and marginally increased iNOS. Progesterone treatment significantly decreased transcription of TNFα, TNFR1, CD11b and TLR4 mRNAs (all *$p<0.05$ vs EAE) and marginally decreased iNOS mRNA. Data derived from Garay et al. [58] .

A CD11b

B TLR4

C iNOS

D TNFα

E TNFR1