Physiological Mini Reviews

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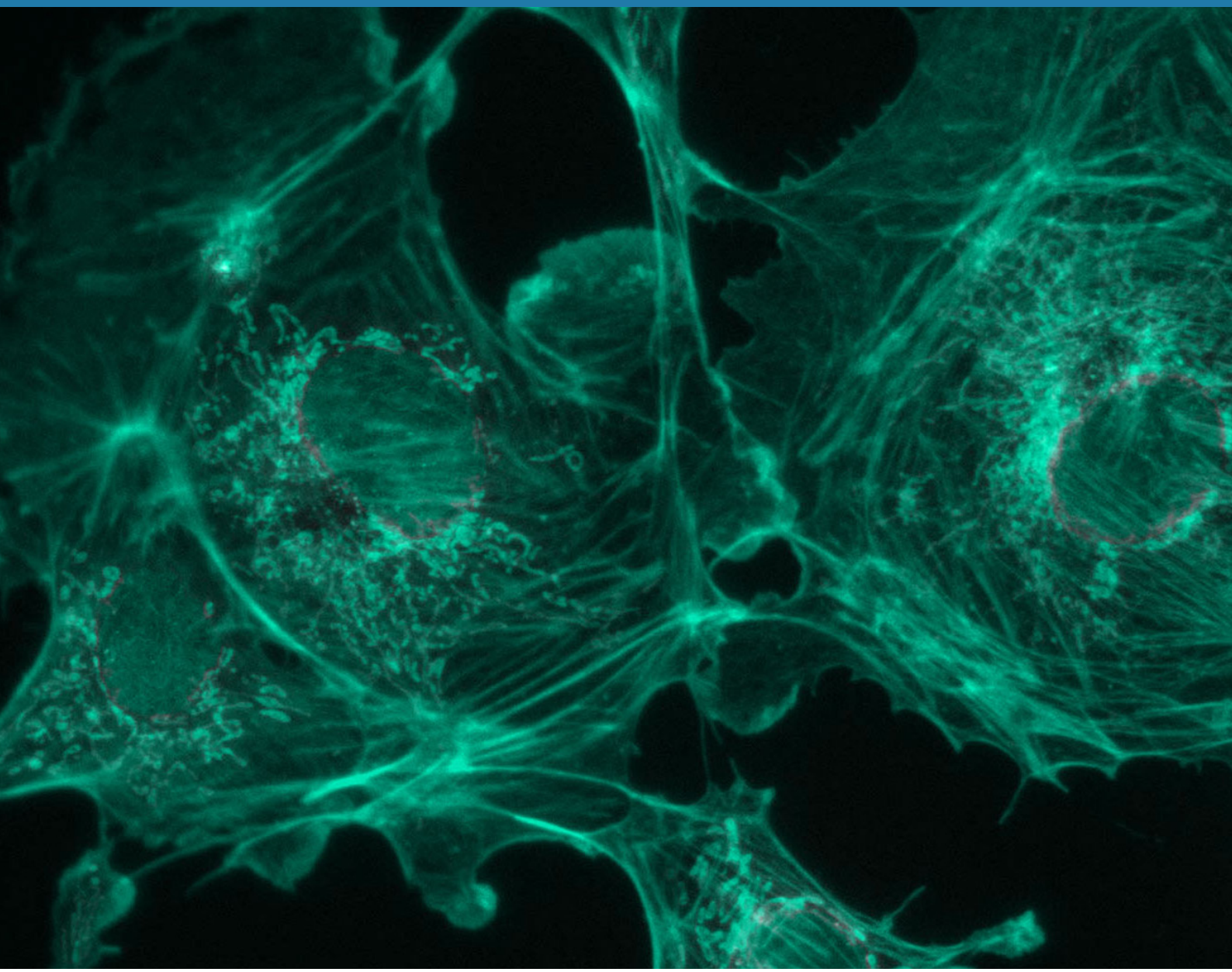

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CELLULAR AND MOLECULAR BASIS OF PROGESTERONE-INDUCED NEUROPROTECTION

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ABSTRACT
Progesterone exerts several effects in the central nervous system not directly involved in reproduction or sex behavior. Non-reproductive effects are better observed under pathological conditions, and include stimulation of myelin formation, neurogenesis and neurosteroidogenesis, preserved mitochondrial function, neuroprotection, anti-inflammatory effects, decreased glutamate excitotoxicity, and regulation of mood, memory and cognition. In addition, the progesterone reduced derivative allopregnanolone shows anxiolytic, sedative and anesthetic properties after binding to GABAa receptors. In the present report we provide examples of [1] progesterone effects on the local synthesis of steroids ("neurosteroids") in a demyelination model, [2] the requirement of the classical progesterone receptor for the anti-inflammatory effects in mice with spinal cord injury, and [3] the protective role of progesterone and allopregnanolone in a mouse model of neurodegeneration. In conclusion, the beneficial effects observed in different experimental paradigms support the versatile properties of progesterone in animal models of central nervous system disorders.
Keywords: Progesterone; neuroinflammation; neurodegeneration; neuroprotection.

RESUMEN
La progesterona produce varios efectos en el sistema nervioso central no relacionados a la reproducción o comportamiento sexual. Estos efectos adicionales se observan preferentemente bajo condiciones patológicas, e incluyen la estimulación de la formación de mielina, la neurogenesis y neuroesteroidogenesis, el mantenimiento de la función mitocondrial, efectos anti-inflamatorios, disminución de la excitotoxicidad del glutamato y regulación del humor, memoria y conocimiento. En agregado, el derivado reducido de progesterona – alopregnanolona – muestra propiedades ansiolíticas, sedantes y anestésicas luego de su unión al receptor GABAa. Este Minireview detalla los efectos de progesterona sobre [1] la síntesis local de esteroides ("neuroesteroides"), en un modelo de desmielinizacion, [2] ejemplifica el requerimiento del receptor clásico de progesterona para los efectos anti-inflamatorios en un modelo de injuria espinal en ratón, y finalmente [3] discute el rol protector de la progesterona y de la alopregnanolona en un modelo murino de degeneración de motoneurona. Como conclusión, los efectos beneficiosos mostrados en diferentes paradigmas experimentales apoyan las propiedades versátiles de la progesterona en modelos de patologías del sistema nervioso central.
Palabras clave: Progesterone; neuroinflammation; neurodegeneration; neuroprotection.

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Introduction

Progesterone is a hormone that plays a pivotal role in reproduction. The isolation of high levels of progesterone from the corpus luteum, ovary and placenta plus the biological activity exerted in reproductive tissues, made progesterone a prototypical sex steroid [1].

A new role emerged for progesterone after discovering its potent actions in the central nervous system (CNS) away from reproduction. A non-reproductive effect of progesterone was first shown by Hans Selye in 1941, after discovering progesterone´s induced anesthesia in the rat. Later on, it was shown that progesterone and its metabolites are synthesized in the brain, in an endocrine-like manner. The local synthesis of steroids in the CNS has been described by the French scientist Emile Etienne Baulieu [2], who baptized them "neurosteroids". Baulieu found that the brain content of certain steroids remains after ablation of peripheral endocrine glands, and that their brain concentration in some cases outnumbered their blood concentration. The CNS not only synthesizes neurosteroids, but also has the capacity to metabolize them. For example, progesterone is reduced to 5 alpha-dihydroprogesterone (abbreviated DHP) and 3 alpha, 5 alpha-tetrahydroprogesteroe (or allopregnanolone, abbreviated ALLO). ALLO exhibits potent anxiolytic and anaesthetic properties after binding to GABAa receptors. Thus, the reported steroid anaesthesia could be explained by progesterone metabolites endowed with membrane-active properties. Additionally, progestins produce many other effects unrelated to reproduction, involving neuroprotection, myelin formation, neurogenesis, control of inflammation, regulation of glial cell function, neurotransmission, growth factor expression, cognition, anti-nociception and ion homeostasis, among others [3].

This Minireview highlights the multifactorial mechanisms of progesterone signalling exemplified in three different models: (a) progesterone regulation of neurosteroidogenesis in multiple sclerosis (MS) mice; (b) the role of the progesterone receptor (PR) in neuroprotection after spinal cord injury, and c) the effects of progesterone and the ALLO derivative in motoneuron degeneration. A prior introduction is given about the different progesterone receptors and the pathways of steroid synthesis in the CNS.

Progesterone signalling in the CNS: role of classical receptors, membrane receptors and the reduced derivatives

Progesterone effects are mediated by several types of progesterone receptors (PR). PRs include the classical nuclear receptors, the membrane receptors mPRs and the progesterone receptor membrane component 1 (PGRMC1). (Figure 1). The "classical" PR is a protein acting as a ligand-dependent transcription factor. The PR is composed of four domains, namely the carboxyl terminal ligand domain, a hinge region, a DNA binding domain and an N-terminal region. The PR exists in two isoforms, PRA and PRB, which are products of a single gene but arising from alternative initiation codons driven by different promoters. PRB contains an additional 164 amino acid called the "B-upstream segment" (BUS) that confers an activation function (AF3). There are two additional ATFs : ATF1 located in the N-terminal domain and responsible for ligand-independent activity and ATF2 that mediates ligand-dependent PR activation. Of the two isoforms, PRB seems a more potent transactivator of gene expression than PRA. PRB immunoreactivity has been localized in neurons, glial cells and ependymal cells. In many of these cell types, PR exists in cytoplasmic and nuclear forms. However, extranuclear PR has also been reported in the pre and postsynaptic areas of hippocampus and motoneurons of the spinal cord [4].The PR dimerizes and binds to sequences called "progesterone receptor-responsive elements" on the DNA of target genes, These are specific

palindromic DNA sequences of bases located in promoters of PR regulated genes. There are also PR monomers acting through "half-site" elements on some target genes. Here, there is a dilemma because the PR and the glucocorticoid receptor (GR) share identical responsive elements on the DNA. Efforts have been made to elucidate how biological diversity between a progestin and a glucocorticoid can be maintained when the activated receptor complexes binds to a common responsive element. The answer comes from (a) different changes of chromatin structure produced by PR or GR, and (b) a role of receptor coactivators and coregulators which are differently recruited depending if the binding protein is PR or GR [5].

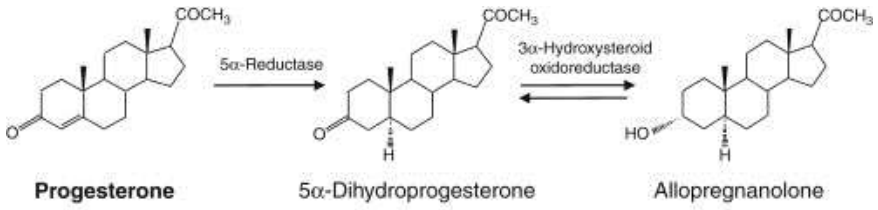
PRs are widely distributed in both brain and spinal cord. In some regions estrogens increase and progesterone down-regulates PR levels, whereas in others the PR is constitutively expressed and does not respond to estrogens. Estrogen-independent PRs are found in the cerebral cortex, septum, caudate putamen, supraoptic nucleus, dentate gyrus, the CA3 subfield of the hippocampus, midbrain, cerebellum and spinal cord. In these regions progesterone treatment does not down-regulate PRs, a mechanism important when seeking a prolonged neuroprotection. Besides the nuclear role of the PR,a specific motif in this protein has been identified that interacts with the SH3 domain of the Src tyrosine kinase. In this mechanism, first described in breast cancer cells, the interaction of a cytoplasmic form of PRB with Src activates the MAPK and ERK signalling pathways [6]. Thus, changes of gene expression could be triggered after interaction of Src with PRB, bypassing the direct transcriptional activity of nuclear PR. In addition, PRs outside the hypothalamus are found in axons, dendrites, and synapses, suggesting that PR located in extranuclear sites interact with plasma membrane proteins. However, the role of the MAPK/ERK pathway in progesterone effects in the brain and spinal cord is not yet clear.

Another "receptor" called PGRMC1 (formerly known as 25Dx), was first isolated from porcine liver and later found in the brain and spinal cord [7,8]. In the CNS it is present in the hypothalamus, circumventricular organs, epdendymal cells, meninges, dorsal horn neurons and ependymal cells of the central canal. There is no total consensus regarding the function of PGRMC1. Some authors consider it a chaperone of a binding protein called serpin mRNA binding protein, others have postulated that PGRMC1 transports PRA to the cell membrane and still others have shown colocalization with the Sigma2 receptor. The detection of PGRMC1 in regions controlling osmoregulation have open new roles for this protein [8]. In rats with spinal cord transection, levels of PGRMC1 mRNA remained unchanged compared to sham-animals, but in rats receiving progesterone treatment PGRMC1 mRNA levels were significantly increased, suggesting that the neuroprotective effects of progesterone obtained following injury may partly involve PGRMC1 in addition to the classical PR [(7].

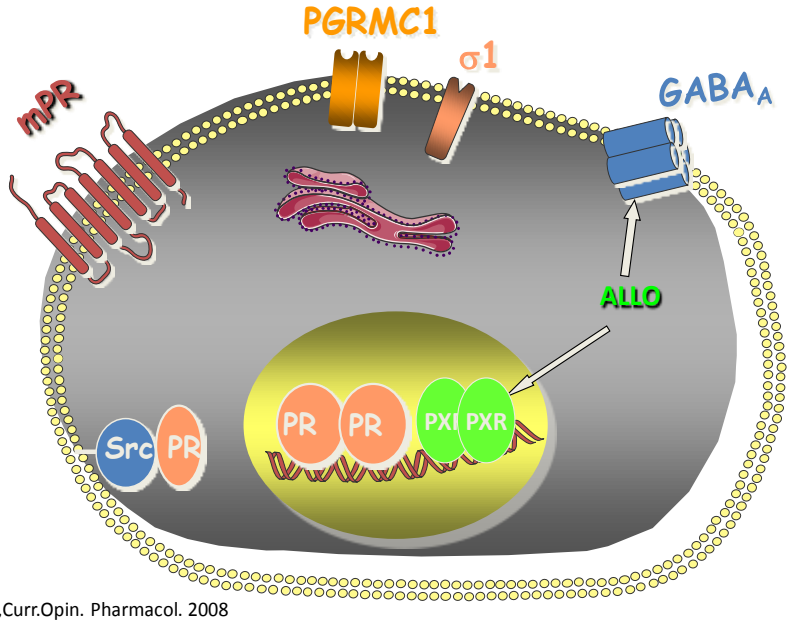
Besides PRA, PRA and PGRMC1, other receptors may account for some progesterone effects. Recently, three isoforms of a membrane receptor for progesterone (mPR) have been cloned from fish and the brain of mammals. These mPRs contain seven integral transmembrane domains, mediate signaling via an inhibitory G-protein coupled pathway and increase the MAPK pathway [6]. In the mouse spinal cord, RT-PCR analysis and sequencing of the amplified products demonstrates the expression of mPRα, mPRβ and mPRγ mRNA, suggesting that mPR receptors may be implicated in some of the biological effects of progesterone in the spinal cord [9]. In this sense, mPRalpha is expressed in most neurons, astrocytes, oligodendrocytes, and also in a large proportion of NG2+ oligodendrocyte progenitor cells. This mPR isoform is thus likely to play a role in the neuroprotective and promyelinating effects of progesterone. On the contrary, mPRbeta was mainly present in ventral horn motoneurons and

in neurites, consistent with a role in neuronal transmission and plasticity. Interestingly, mPRbeta was not present in glial cells.

The molecular mechanisms of progesterone effects are open to question. The lack of a responsive element for the PR on the promoters of some proinflammatory genes (checked by Alibaba 2.1 transcription factor binding prediction program, available at http://www.gene-regulation.com/pub/programs/alibaba2/index.html) suggests transrepression mechanisms: such as the interaction of PR with the AP1 protein (a JUN /FOS complex) and binding of the complex to AP-1 sites on DNA, or repression of NFκB transactivation, since PR is the only steroid receptor besides the GR able to repress NFκB mediated transcription. Progesterone-bound PR can directly repress the COX2 gene in endothelial cells, and regulate in a negative fashion iNOS expression by macrophages. Since iNOS and COX-2 are also stimulated by IL1β and TNF-α, there may be indirect effects of progesterone on these enzymes due to a decrease in those cytokines. Whether non-classical receptors including mPRs, the $GABA_A$, PRCM1, Sigma1 or PXR mediate progesterone anti-inflammatory effects is also intriguing because PRKO mice were unresponsive to progesterone. PRKO mice normally express these alternative progesterone mediators, but they lack progesterone immunosupression. Perhaps PR also brings an adequate microenvironment for the function of the mentioned molecules in the inflamed CNS. Elucidation of the multiple anti-inflammatory mechanisms of progesterone remains an exciting challenge of future experiments.



Progesterone and 5α-dihydroprogesteroine bind to the classical progesterone receptor (PR), mPRs and PGRMC1, whereas ALLO binds to other molecules but not to PR.



Schumacher et al.,Curr.Opin. Pharmacol. 2008

Figure 1: Top structures show progesterone and its metabolites 5α-dihydroprogesterone and 3α,5α-tetrahidroproegsterone (allopregnanolone). The bottom image shows progesterone receptors and binding molecules in the nervous system. Besides binding to the classical nuclear receptor (PR) that binds to sequences on the DNA and regulates transcription, PR can activate extranuclear signaling pathways after protein-protein interaction with the Src family of proteins. Progesterone also binds to several types of membrane receptors (mPR) associated with G proteins, and regulate intracellular calcium after binding to the progesterone receptor membrane component 1 (PGRMC1). The reduced derivative allopregnanolone (ALLO) binds to GABAa receptors, Sigma1 receptor and pregnane X receptor (PXR), influencing different signaling mechanisms. (Modified from ref. 10).

Pathway of progesterone biosynthesis in the CNS:

Neurosteroidogenesis is the synthesis de novo of steroids (neurosteroids) by the CNS and the peripheral nervous system. It is a dynamic process showing fluctuations under physiological conditions such as brain development, ovarian cycle and pregnancy. However, marked changes of neurosteroids have been reported in pathological conditions, including psychiatric disorders, Alzheimer´disease, Parkinson´s disease, multiple sclerosis, Nieman-Pick type C disease, diabetic neuropathy, peripheral neuropathy, traumatic brain injury, spinal cord injury and ischemic stroke [11 – 14]. Therefore, it is likely that neurosteroids play important roles in both the normal and pathological CNS.

The pathway of neurosteroidogenesis resembles the pathway of steroidogenesis in peripheral endocrine glands. It involves translocator proteins and enzymes responsible for the formation of progesterone and reduced derivatives, androgens and estrogens. Neurosteroids can be produced by neurons, astrocytes, oligodendrocytes and Schwann cells [14]. 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 of peripheral endocrine glands. 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). Once inside the mitochondria, the cholesterol side chain cleavage enzyme (P450scc) splits the side chain of cholesterol converting it into pregnenolone.

In the following steps, pregnenolone is metabolized to progesterone, which becomes the mother molecule for the synthesis of reduced derivatives (DHP, ALLO) and also androgens and estrogens. The enzymes catalyzing these conversions are found in the endoplasmic reticulum (ER). For example, metabolism of progesterone into DHP by 5α-reductase is present in oligodendrocytes and astrocytes , whereas 3α-hydroxysteroidoxireductase (3α-HSOR) is present in oligodendrocytes, astrocytes and neurons [14]. These cells metabolize DHP into the potent GABA(A) receptor agonist ALLO [15]. Furthermore, progesterone is also metabolized into androgens and estrogens. The enzyme aromatase that converts androgens into estrogens is normally detected in the endoplasmic reticulum of neurons, but becomes highly expressed in astrocytes after brain injury or in Alzheimer`s disease. Figure 2 shows the pathways of neurosteroidogenesis from cholesterol into progesterone and derivatives. Several of these steroid products are found in the CNS [16]. Neurosteroidogenesis is under regulatory control by many factors, which generally increased one or more of the protein or enzymatic steps:

Steroids: estradiol, progesterone, dexamethasone, testosterone, steroidogenic factor 1

Steroid receptors. PGRMC1, PXR

Neurotransmitters: endocannabinoids, NMDA, CAMP-stimulated pathways

Pathological conditions: CNS injury, peripheral nerve injury, diabetes mellitus

Other factors: TSPO ligands, ethanol, 9-cis-retinol.

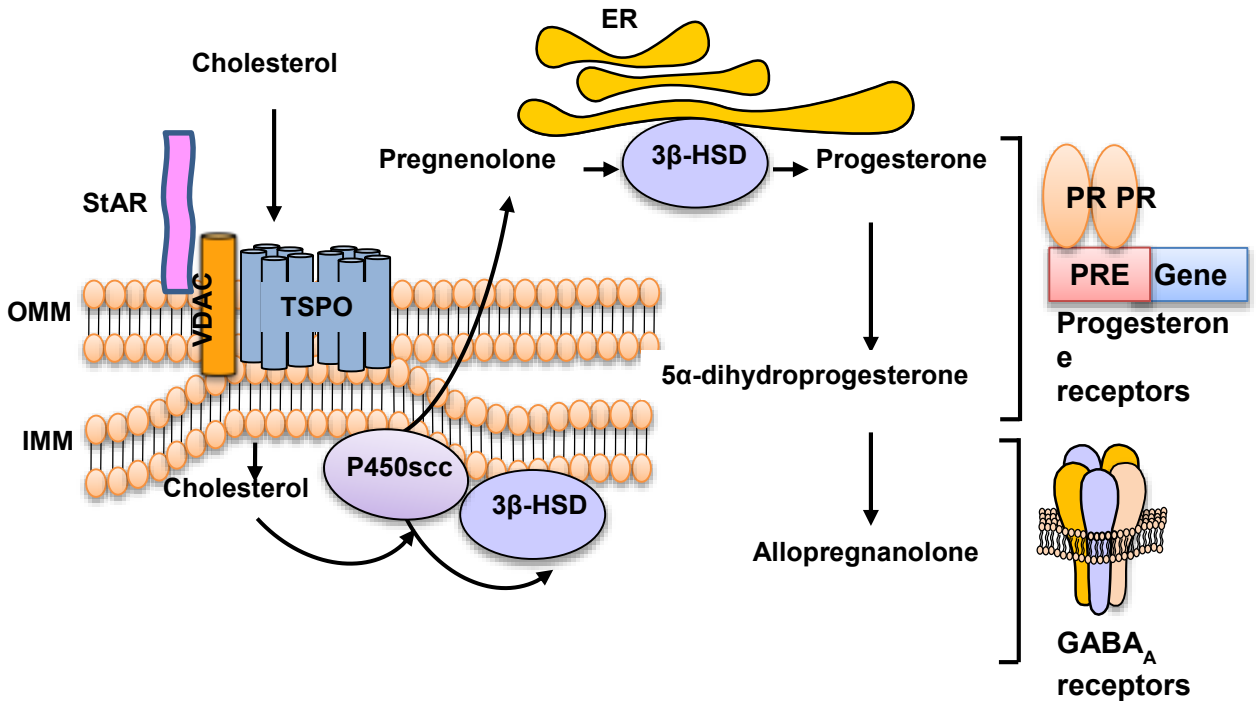


Figure 2: Diagram showing the synthesis of neurosteroids. Cholesterol enters the mitochondria due to a multiprotein complex composed by the proteins StAR (steroidpgenic acute regulatory protein), translocator protein 18 KD (TSPO), voltaje-dependent anion channel (VDAC) and other associated proteins. Once inside the mitochondria, cholesterol is converted into pregnenolone by the cholesterol side-chain cleavaje enzyme (P450scc). Pregnenolone leaves the mitochondria and is metabolized to progesterone by the 3 beta-old dehydrogenase. Progesterone in turn is reduced to 5α-dihydroprogesterone (DHP) and DHP reduced by 3α-steroid oxidoreductase (3α-SOR) to allopregnanolone (ALLO). Progesterone and DHP bind to progesterone receptors, Allopregnanolone is a GABAa receptor agonist (Modified from ref. 6 and 10).

Effects of progesterone on neurosteroidogenesis in a mouse model of multiple sclerosis (MS).

In this section, we discuss the possibility that stimulation of neurosteroidogenesis may be beneficial for CNS diseases. To test this hypothesis, we employed an animal model of MS. MS is a neurological disorder that strikes the spinal cord, brain and optic nerves with a female to male incidence of 2:1. In about 80% of the patients, it shows a relapsing-remitting course [17]. Based on this outcome, MS has been considered of autoimmune origin, showing inflammatory infiltrates, activated microglial cells and increased proinflammatory factors that cause loss of oligodendrocytes, demyelination and impair axonal conductance. A role for steroid hormones in MS is suggested based on the high female to male ratio and because relapses decline during the last trimester of pregnancy, when estrogens and progesterone levels are high, and resume when steroids decay after delivery [18]. Measurements of circulating and brain steroid levels reinforce the participation of steroids in MS. Thus, MS patients show reduced levels of ALLO and of the enzymes converting progesterone into DHP and ALLO, and also show changes of progesterone levels in cerebrospinal fluid and plasma [18]. In turn, progesterone, a synthetic progestin and ALLO produce beneficial effects in experimental autoimmune encephalomyelitis

(EAE) and in the cuprizone model, two widely used procedures to induce MS in mice and rats [13, 18, 19].

For our studies, we used female C57BL/6 mice with EAE induced by a myelin oligodendrocyte glycoprotein peptide (MOG_{40-54}). We found that progesterone treatment enhances myelin formation, decreases inflammation and stimulates neurosteroidogenesis. Nine-10 days following MOG administration mice developed clinical signs of EAE (i.e. loss of tail tonicity, paralysis,etc) and showed inflammatory cell infiltration and demyelination with reductions of the major central myelin proteins: myelin basic protein (MBP) and proteolipid protein (PLP) in the spinal cord. In this pathological environment, progesterone pretreatment showed less inflammatory cell infiltration, recover myelin proteins, delayed the onset of the disease and attenuated clinical scores. Protective effects were also obtained with the synthetic progestin Nestorone, an analog showing high affinity for the PR.

These sets of experiments clearly showed that neurochemical abnormalities of untreated EAE mice were accompanied by diminished neurosteroidogenesis (Figure 3). Thus, EAE mice showed decreased mRNA expression of StAR, VDAC, P450scc cholesterol side-chain cleavage, 5 α-reductase, 3α-HSD and aromatase in the spinal cord. Instead, mRNA levels of 3β-hydroxysteroid dehydrogenase (3β-HSD) showed a large intergroup variation. We also found increased mRNA expression of TSPO, which likely originated on the reactive microgliosis of EAE mice. These changes were reversible, because pretreatment with progesterone increased StAR, VDAC, P450scc, 5α-reductase, 3α-HSD and aromatase mRNAs and did not modify 3β-HSD (Figure 3). TSPO mRNA was decreased, consequent with inhibition of microgliosis.

Concomitantly, progesterone blocked the EAE-induced increase of the proinflammatory mediators tumor necrosis factor alpha (TNFα), its receptor TNFR1, the toll-like receptor 4 (TLR4) mRNAs, and increased the expression of the central factors needed for oligodendrogenesis and myelinogenesis (NKx2.2 and Olig) and enhanced the number of oligodendrocytes labeled with the CC1 antibody [13] . Progesterone treatment decreased the number of Iba1+ microglial cells and the microglial marker CD11b, in addition to the mentioned blockage of proinflammatory factors in EAE mice [13]. These results support an anti-inflammatory role of progesterone in EAE mice. This mechanism strongly resembles the immunomodulatory effects of progesterone during pregnancy, in which a change occurs from the Th1 pro-inflammatory to a Th2 anti-inflammatory response.

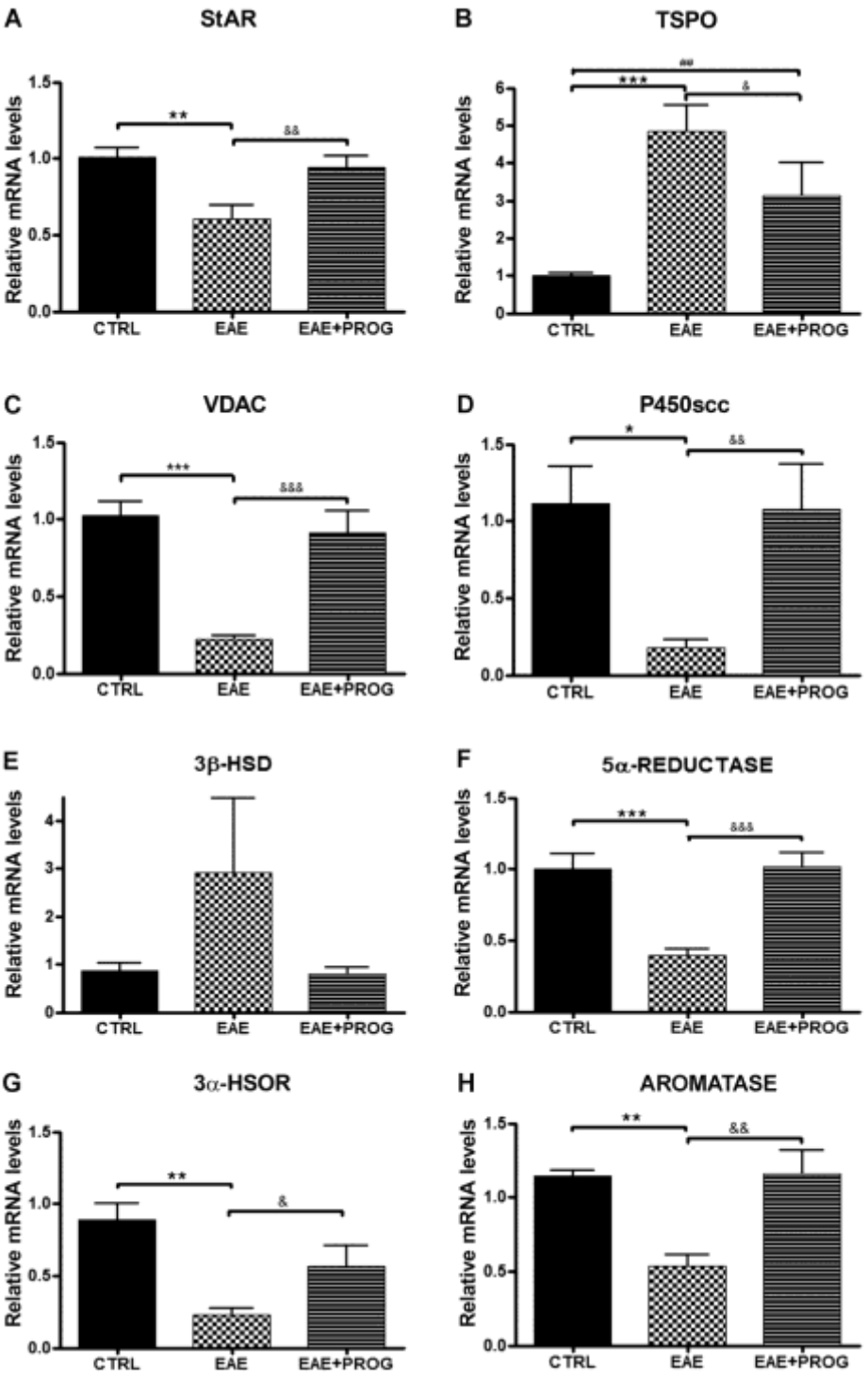


Figure 3: mRNA levels of the transduceosome and microsomal neurosteroidogenic enzymes in the spinal cord from control, EAE and EAE mice receiving progesterone (PROG) pretreatment. A: Levels of Star were decreased in EAE mice (** $p<0.01$ vs control) and increased in the EAE + PROG group (&& p0.01 vs EAE). B: 18 K (TSPO) mRNA was increased in EAE (*** $p<0.001$) and decreased in EAE+PROG mice ($^{\&}$ $p<0.05$). The mRNA of the EAE+PROG group remained higher than control ($^{\#\#}$ $p<0.01$). C: Levels of VDAC) were decreased in EAE (***$p<0.001$) and increased in EAE+PROG mice

($^{\&\&\&}p<0.001$). D: mRNA P450scc was decreased in EAE ($^{*}p<0.05$) and increased in the EAE+PROG mice ($^{\&\&}p<0.01$). E: Variations of the mRNA of (3β-HSD were not significant different between control, EAE and EAE+PROG groups. F. mRNA for 5α-reductase were decreased in EAE ($^{***}p<0.001$) and increased in EAE+PROG-treated mice ($^{\&\&\&}p<0.001$). The last group was not different from controls (p:NS). G: mRNA of 3α-HSD was decreased in EAE ($^{**}p<0.01$) and increased in the EAE+PROG group ($^{\&}p<0.05$). The low mean of the last group was not significant different from controls (p:NS). H: Aromatase mRNA was lower in EAE ($^{**}p<0.01$ vs. control) and increased in the EAE+PROG-treated mice ($^{\&\&}p<0.01$). The last group and control measured similarly. Statistical analysis obtained by ANOVA followed by the Newman-Keuls test (n= 6-7 animals per group). (Modified from ref. 13).

We have already discussed that the spinal cord express several types of receptors for progesterone (PR, mPRs, PGMC1) and binding molecules for the reduced derivatives (GABAa receptor, PXR, Sigma1 receptor), although factors involved in the anti-inflammatory and promyelinating effects in EAE have not being clarified. Use of mice with a PR deletion did not give an answer, because the PRKO strain was resistant to EAE induction. However, the fact that the synthetic progestin and agonist of the PR Nestorone brings protection to EAE mice supports a role of the nuclear receptor (20). Whatever the operating mechanism, however, stimulation of neurosteroidogenesis might provide an extra source of progesterone or ALLO to the inflamed nervous tissue. In this case, neurosteroids may work in an autocrine or paracrine fashion, amplifying the effect of systemically-given progesterone. Thus, part of the beneficial effects of progesterone in the EAE model may be due to enhanced neurosteroidogenesis. Other reports have more firmly established that pharmacological stimulation of neurosteroidogenesis with TSPO and analogs improves the clinical outcome and neuropathology of EAE mice and also of rats with diabetic neuropathy [21, 22]. Thus, the possibility that neurosteroidogenesis can be stimulated opens new therapeutic perspectives for CNS diseases.

Role of the progesterone receptor in the recovery from spinal cord injury (SCI)

This section discusses if the PR is a required partner for progesterone effects on SCI. SCI is a devastating incident that targets all cells of the lesioned tissue. Neurons suffer necrosis, apoptosis, oxidative damage, chromatolysis, axonal demyelination and functional impairment. Oligodendrocytes die by apoptosis and the damaging effect of proinflammatory mediators. Instead, astrocytes and microglia become reactive and produce proinflammatory mediators, oxygen free radicals and neurotoxic levels of NO as part of a process known as reactive gliosis. Reactive astrocytes and microglia release proinflammatory mediators which reciprocally regulate each other, producing a feed-forward mechanism that propagates secondary injury and inflammation after spinal cord injury

In mice with SCI, there is a fast response of the acute-phase proinflammatory cytokines IL1β, IL6 and TNFα released from reactive astrocytes and microglia. SCI also increased the expression of the pro-inflammatory enzymes inducible nitric oxide synthase (iNOS) and cyclooxigenase 2 (COX2). Induction of NOS in astrocytes and microglia produces neurotoxic levels of NO, which can impair mitochondrial respiration, block the electron transport carriers and increase the synthesis of reactive oxygen species. COX2 converts arachidonic acid into prostaglandins, which are strong oxidants, increasing peroxidation of membrane lipids and resulting in cell death. Therefore, excess production of IL1β, TNFα, IL6, iNOS and COX-2 jointly contribute to white matter demyelination and oligodendrocyte loss reported in SCI [23]. In this disturbing environment, therapies aimed at blocking the innate immune response and holding back the glial reaction may be relevant for preserving functional loss. Successful trials with progesterone in animals with SCI encouraged studies of the mechanisms involved in

progesterone neuroprotective, promyelinating, anti-inflammatory and anti-nociceptive effects [3]. To show the role of the PR in progesterone effects in SCI, we employed PRKO mice (PR^{lacz} mice on a C57BL6/129SvEv background) and wild- type mice. The PRKO mice lack both PRA and PRB isoforms. The mRNA levels of IL1β, TNFα, and IL6 were assessed in wild-type and PRKO mice 6 h after SCI or sham operation with or without progesterone treatment (Figure 4). In brief, SCI strongly upregulated IL1β, TNFα, and IL6 mRNA; interestingly, the increase in the cytokines occurred for both wild-type and PRKO mice. However, the response to progesterone highly diverge between wild-type and PRKO mice. Whereas in wild-type mice progesterone significantly inhibited the mRNAs of IL1β, TNFα and IL6, the steroid was totally ineffective in the PRKO mice (Figure 4). These data strongly supported the need for a functionally intact PR for progesterone´ s anti-inflammatory effects after SCI [23].

To test whether changes in the expression of cytokines and proinflammatory enzymes in response to SCI and progesterone treatment reflected changes in glial cell reactions, astrocytes and microglial cells were counted at 6 h post-injury by computerized stereology. SCI produced a strong astrogliosis and microgliosis in both wild-type and PRKO mice. Six hours after progesterone treatment of injured wild-type mice, both astrogliosis and microgliosis were significantly down-regulated. In contrast to wild-type mice, progesterone treatment failed to decrease glial cells in the PRKO group. Thus, SCI stimulated astrogliosis and microgliosis in both mice with intact or nonfunctional PR, whereas progesterone treatment reduced the number of reactive astrocytes and microglia in wild-type but not PRKO mice. Furthermore, the PR was also required for the survival of oligodendrocyte precursor cells (OPC) labelled with the NG2 marker. After injury, mature oligodendrocytes are lost and the remaining ones do not divide. Oligodendrocyets are then replaced by a wave of OPC differentiating into myelin-producing oligodendrocytes. To investigate if progesterone increase of OPCs is due to blockage of apoptosis, we counted double-labeled apoptotic OPCs, localized by being TUNEL+ and NG2+ (double labeled cells). Following SCI, we found that in wild-type mice progesterone reduced the % of double labeled cells (i.e. apoptotic cells), but had no effect in the PRKO (Figure 4).

We concluded that progesterone exerted powerful anti-inflammatory effects after SCI and that remyelination of the injured tissue occurred by recruitment of OPCs. Both events required the PR because they were absent in the PRKO mice. The PR-mediated effect of progesterone on the reduction of the interleukins, TNFα and the proinflammatory enzymes might be crucial for the recovery of function following SCI. In this regard, we have already shown that progesterone significantly improved motor outcomes in two different tests of motor behavior. The findings have important translational perspective for patients with SCI.

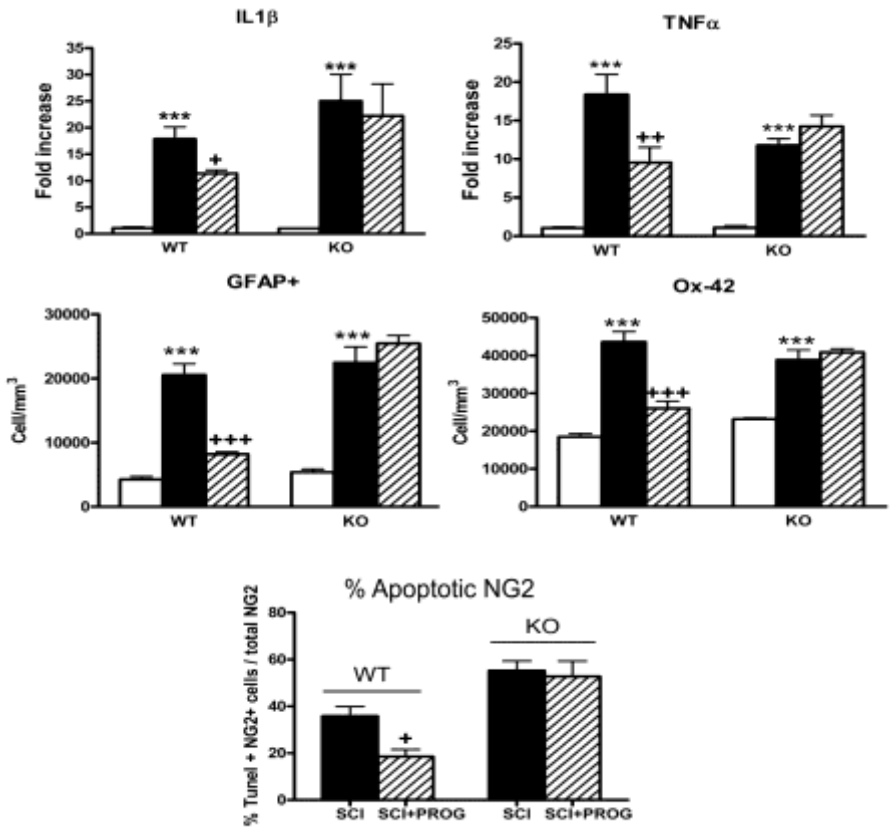


Figure 4: Progesterone decreases proinflammatory mediators and gliosis in wild-type but not in PRKO mice. Spinal cord injury (SCI) increases the mRNA of IL1β, TNFα, the number of GFAP+ astrocytes and of OX-42 labeled microglia in both wild type (WT, dark columns) and PRKO (KO, columns with crossing lines) vs. control mice (*** $p<0.001$ for all). Progesterone treatment decreased IL1β in wild type mice (*<0.05 vs SCI), TNFα ($^{++}$ $p<0.01$ vs SCI), GFAP+ astrocytes ($^{+++}$ $p<0.001$ vs SCI) and OX-42 microglia ($^{+++}$ $p<0.001$ vs SCI), but was inactive on these parameters in PRKO mice. The bottom graph shows that progesterone decreased apoptosis of NG2+ oligodendrocyte precursors in wild type ($^{+}p<0.05$ vs SCI) but not in PRKO. n= 8 animals per group (modified from ref .23).

Protective role of progesterone and allopregnanolone in degenerative and other CNS diseases

Literature reports, including our own, suggest a director protective effects of progesterone after binding to intracellular PRs. However, treatment with the reduced derivative ALLO also lead to beneficial effects. ALLO protective effects have been demonstrated in brain trauma and ischemia, Niemann-Pick type C disease (a lipid storage disease), Parkinson´s disease and multiple sclerosis (24 ,25). In a review paper by Guennoun et al. [24], the authors described that ALLO effects include the inhibition of the mitochondrial permeability transition pore, prevention of apoptosis, maintenance of the blood-brain barrier, reduction of neuroinflammation, and shrinking of infarct size. In vivo, ALLO treatment provides neuroprotection in a transgenic mouse model of Alzheimer's disease (3xTgAD mice). Thus, in young 3xTgAD transgenic mice and wild type old mice treatment with ALLO increases hippocampal neurogenesis, induces phosphorylation of CREB (cyclic AMP response element

binding protein) and stimulates the levels of neuronal differentiation transcription factors. The 3xTgAD mice also show restoration of learning and memory. At the cellular level, ALLO increases the expression of the 2′ 3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase), a specific marker of oligodendrocytes and reduces amyloid-β peptide generation and neuroinflammation . Finally, ALLO induces the proliferation of rat and human hippocampal neural stem cells in culture.

Besides the abovementioned diseases, ALLO treatment resulted effective for neurodegeneration. In our studies, we employed the degenerating spinal cord of the Wobbler mouse as a model to test ALLO protective effects. The Wobbler is a murine model of amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disease characterized by the selective and progressive death of both upper and lower motoneurons, leading to a progressive paralysis of voluntary muscles, respiratory failure and death in less than 5 years. The Wobbler mice suffer a spontaneous mutation of the vacuolar/vesicular protein sorting 54 (Vps 54) gene leading to motoneuron degeneration in motor cortex, brainstem and cervical spinal cord. Neuronal alterations associate with increased oxidative stress, mitochondrial dysfunction with failure of the respiratory chain complexes, reduction of the cholinergic enzyme choline acetyl-transferase (ChAT) and low expression of brain derived neurotrophic factor (BDNF) in motoneurons, impairment of slow axonal transport and gait disturbances [26]. Wobbler mice also present intracellular ubiquitin inclusions, abnormal distribution of transactive response DNA binding protein-43 (TDP-43) into the cytoplasm, cortical hyperexcitability and positive response to the anti-glutamatergic drug Riluzole, in similarity with people with ALS. First, we produce substantial evidence that administration of progesterone to Wobbler mice reduces morphological, molecular and functional abnormalities of motoneurons and glial cells and increases muscle strength and life span. In these mice receiving progesterone, there is high levels of plasma and tissue ALLO, suggesting this compound as a mediator of progesterone protective effects [16]. This conclusion prompted additional studies to find out if ALLO treatment delayed the progression of spinal cord degeneration.

Among the pathogenetic defects of Wobbler mice, increased oxidative stress is at the frontline. This abnormality predominates in neurons at the early symptomatic stage, as indicated by intense vacuolation, increased generation of free radicals, 4-hydroxynonenal and the high activity of nitric oxide synthase (NOS). The attenuation of motoneuron abnormalities by antioxidant agents, nitric oxide inhibitors, the antioxidant steroid U-74389F and Edaravone, a free radical scavenger also support an important role of oxidative stress in Wobbler´s motoneuron pathology [26, 27]. In clinically affected Wobbler mice, treatment with ALLO reduces neuronal vacuolation and the activity of the nitric oxide synthesizing enzyme (NOS) both in neurons and also glial cells. These effects are shared by both progesterone and ALLO. We speculated that progesterone binding to PR in spinal cord motoneurons protects mitochondria from toxic levels of NO by down-regulation of NOS activity. On the other hand, ALLO might regulate neuronal NOS per se, after activation of $GABA_A$ or by inhibition of glutamate NMDA receptors [28]. For instance, application of ALLO to developing neuronal cells increases α4 $GABA_A$ receptor subunit. ALLO also increases delta $GABA_A$ receptor subunit, which is important for neurosteroid modulation of tonic inhibition. This is a major mechanism to control excitatory synapses and reduce hyperexcitability of motoneuron. Furthermore, ALLO also blocks glutamate excitotoxicity and, consequently, Ca^{++} dependent activation of nNOS in motoneurons. The latter effect decreases intracellular Ca^{++} and, in consequence, Ca^{++}-dependant activation of neuronal NOS.

However, NOS reduction of Wobbler motoneurons does not persist for a long time following ALLO treatment, in contrast to NOS activity of glial cells which lasts longer. Therefore, ALLO inhibitory effects on NOS may differ in time in motoneurons (early effect) and glial cells (late effect). This dual effect will be important for neuroprotection. It should be reminded that NO is a free radical because it contains an unpaired electron; blockage of NO synthesis by ALLO may stop the propagation of nitrergic stress, peroxinitrite formation and mitochondrial damage. However, the issue seems more complex than previously thought. ALLO protective effects are probably multifactorial, since it *(1)* regulates the alpha and delta $GABA_A$ receptor subunits and the activity of this neurotransmitter receptor, *(2)* binds to the pregnane X receptor (PXR), (3) interacts with the membrane PR (mPR), and *(4)* interacts with the Sigma1 receptor (5) , as mentioned before.

The diverse effects shown for ALLO in animal models or cultured cells push forward several clinical studies (http://clinicaltrials.gov) using ALLO or its analog Ganaxolone (3β-Methyl-5α-pregnan-3α-ol-20-one; 3α-Hydroxy-3β-methyl-5α-pregnan-20-one). Trials have been done or are in course for traumatic brain injury, fragile-X-syndrome, mild cognitive impairment due to Alzheimer´s disease or without it, exaggerated stress responses and smoking, premenstrual dysphoric syndrome, depression, chronic pain, anxious thoughts, epilepsy and susc It is thop apy

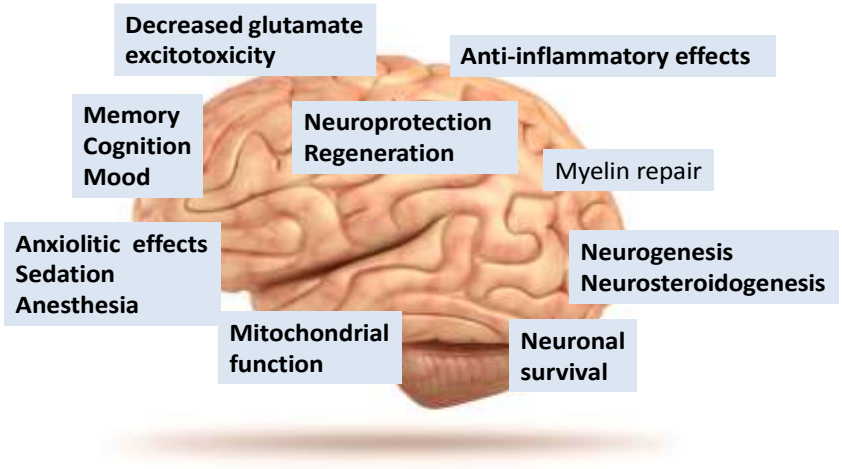


Figure 5: A summary of the effects of progesterone in the CNS. Besides its pivotal effects on the reproductive and neuroendocrine axis, a number of non-reproductive effects have been shown at the experimental level. These include neuroprotection, promyelinating effects, regulation of mitochondrial function, enhanced neuronal survival, stimulation of neurogenesis and neurosteroidogenesis, anxiolytic and sedative effects after bioconversion into ALLO, and consolidation of memory and cognition. On the other hand, progesterone decreases neuroinflammation, lipid peroxidation and glutamate excitotoxicity. Further actions of this multifacetic steroid are expected to emerge in the future for the normal and pathological CNS.

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