Therapeutic Effects of Progesterone in Animal Models of Neurological Disorders

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Abstract: Substantial evidence supports that progesterone exerts many functions in the central and peripheral nervous system unrelated to its classical role in reproduction. In this review we first discussed progesterone effects following binding to the classical intracellular progesterone receptors A and B and several forms of membrane progesterone receptors, the modulation of intracellular signalling cascades and the interaction of progesterone reduced metabolites with neurotransmitter receptors. We next described our results involving animal models of human neuropathologies to elucidate the protective roles of progesterone. We described: (a) the protective and promyelinating effects of progesterone in experimental spinal cord injury, (b) the progesterone protective effects exerted upon motoneurons in the degenerating spinal cord of Wobbler mouse model of amyotropic lateral sclerosis; (c) the protective and anti-inflammatory effects of progesterone in the murine experimental autoimmune encephalomyelitis model of multiple sclerosis and after lysolecithin demyelination; (d) the progesterone prevention of nociception and neuropathic pain which follow spinal cord injury, and (e) the protective effect of progesterone in experimental ischemic stroke. Whenever available, the molecular mechanisms involved in these progesterone effects were examined. The multiplicity of progesterone beneficial effects has opened new venues of research for neurological disorders. In this way, results obtained in animal models could provide the basis for novel therapeutic strategies and pre-clinical studies.

Keywords: Progesterone, neuroprotection, steroid receptors, animal models.

A TALE OF THE STORY

The multiplicity of progesterone effects on the nervous system has opened new venues of research and therapeutic opportunities for neurological disorders. Considered a prototype sex steroid hormone, progesterone regulates gonadotrophin secretion, ovulation, mating behavior and pregnancy by acting at brain centers that control reproductive functions [1, 2]. In the nervous system, however, progesterone exerts many effects unrelated to reproduction. Substantial evidence supports that progesterone action in the nervous system involves neuroprotection, myelin formation, neurogenesis, control of inflammation, regulation of glial cell function, neurotransmission, growth factor expression, cognition, anti-nociception and ion homeostasis, among others [3-5]. Early findings of non-reproductive progesterone actions in the central nervous system (CNS) involved the plasma membrane. In 1941, Hans Selye has discovered steroid anesthesia after injecting a high progesterone dose to a rat [6]. Since the injection put the animal to sleep in a short period of time, the mechanism clearly departed from the classical regulation of gene transcription. Selye`s early finding has heralded later experimental observations by Majewska *et al.* [7] who demonstrated that progesterone´ anesthetic and anxiolytic effects are due to conversion to the ring A reduced metabolite allopregnanolone (3α, 5α-tetrahydroprogesterone). Allopregnanolone, an agonist acting at the GABAa receptor reinforces inhibitory neurotransmission although it is also endowed with neuroprotective activities [8-10]. In addition to GABA neurotransmission, progesterone also modulates N-methyl-d-aspartate (NMDA) and Sigma-1 receptors [11]. In this way, sensitivity to progesterone is enhanced *via* convergence of multiple signaling pathways in the CNS.

In addition to modulation of neurotransmitter receptors, many progesterone effects are mediated by "classical" receptors, acting like ligand-dependent transcription factors. The progesterone receptor (PR) exists in two isoforms, PRA and PRB, which are products of a single gene but arise from alternative initiation codons driven by different promoters [12]. PR has been found in hippocampus, cerebral cortex, hypothalamus, cerebellum and spinal cord [5, 13-15]. Of the

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two isoforms, PRB seems a more potent transactivator of gene expression than PRA [11]. 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 [16, 17]. PRs different from the classical cytosolic and nuclear forms have also been reported in the plasma membrane (mPR), and classified as α, β and γ subtypes. These mPRs contain seven integral transmembrane domains, mediate signaling *via* an inhibitory G-protein coupled pathway and increase the MAPK pathway [18]. mPRα, mPRβ and mPRγ mRNA are expressed in the brain and spinal cord. Whereas motoneurons express mRNAs for PRα and PRβ, only mPRα has been detected in glial cells [13]. Besides the mentioned mPRs, progesterone also binds to the putative membrane progesterone receptor 25DX, presently renamed progesterone receptor membrane component 1 (PGRMC1). The latter is a membrane-bound progesterone binding site that shows several potential signaling mechanisms, including the Jak/STAT and Src pathways and the activation of protein kinase G [19, 20]. To further complicate the issue, in some systems the interaction of extranuclear PR with the Src-ERK1/2 pathway leads to modulation of intracellular signaling cascades and to decreases in the intracellular Ca concentrations [21]. These signaling cascades, at the end, may also regulate transcriptional events, although in a manner different from the classical PR.

Baulieu has shown in 1981 that the brain contained higher concentrations of certain steroids compared to their blood levels. Besides, some steroids accumulated in the brain independently of their adrenal and gonadal sources. These findings led to the discovery of steroid biosynthetic pathways in the CNS not different from those described in the peripheral endocrine glands. Steroids produced in the CNS or "neurosteroids", are synthesized from cholesterol by many cells of the central and peripheral nervous system including neurons, oligodendrocytes, astrocytes and Schwann cells [22]. Regarding progesterone, nervous system cells contain all the enzymatic machinery necessary to synthesize it from cholesterol as well as to metabolize progesterone to 5α-dihydroprogesterone (DHP) and allopregnanolone. However, progesterone synthesized and secreted by the gonads and adrenal glands also acts on the CNS, as the hormone easily crosses the blood-brain-barrier. Functionally, progesterone and its metabolites may be considered as neuroactive steroids, as they modulate the activity of neurotransmitter receptors and ion channels and thus influence neuronal excitability [23].

In this review article, we summarize current work on progesterone effects in different animal models of human neurological disorders. Research in animal models not only improves our understanding of steroid action in nervous system diseases, but could also provide the basis for novel therapeutic strategies and pre-clinical studies.

PROGESTERONE NEUROPROTECTION IN EXPERIMENTAL SPINAL CORD INJURY

Traumatic spinal cord injury is a life crippling event ending in paraplegia, wheelchair support and artificial maintenance of bowel and bladder function. At present, motor vehicle accidents are the most common cause of spinal cord injury in humans [24].

The cells populating the spinal cord respond differently to injury. Neurons are severely compromised and suffer necrosis, apoptosis, degeneration and chromatolysis depending on the modality of damage [25]. At the neurochemical level, parameters indicative of normal neuronal function show negative regulation after acute transection in rats. These include choline acetyltransferase (ChAT), Na, K-ATPase, and brain-derived neurotrophic factor (BDNF), whereas a fourth parameter is stimulated (growth-associated protein (GAP-43). A 3-day course of intensive progesterone treatment of transected rats restores ChAT immunoreactivity, increases the expression of the mRNA for the alpha3 catalytic and beta1 regulatory subunits of neuronal Na, K-ATPase and further enhances the upregulation for GAP-43 mRNA in ventral horn neurons. The progesterone-mediated effects on these three markers are observed in Lamina IX motoneurons, as well as in smaller neurons measuring approximately <500 μm^2. The stimulatory effects of progesterone on ChAT appears to replenish acetylcholine, while its stimulatory effects on Na, K-ATPase seems capable of restoring membrane potential, ion transport and nutrient uptake. Progesterone effects on GAP-43 also appear to accelerate reparative responses to injury, whereas effects on BDNF provide a trophic, reparative response involved in neuroregeneration [3, 25, 26].

It is likely that progesterone may correct these abnormalities by acting directly upon the damaged neurons, as they express several forms of PRs. Immunocytochemistry demonstrated that neurons from ventral horn and Lamina IX are PR-B positive [17]. Further investigations employing real-time PCR supported the existence of PR-B and PGRMC1 in the spinal cord [18]. The response of PGRMC1 to injury, suggest its involvement in the neuroprotective effects of progesterone in the spinal cord and brain [19]. However, other receptors may also play a role in the neuroprotective effects of progesterone after motoneuron injury, because mRNA for mPRα and mPRβ have been found in motoneurons. Thus, the effects of progesterone in the injured spinal cord may result from action at different receptors and signaling pathways, although their specific contributions still remain to be clarified [13, 17, 18].

Oligodendrocytes, the myelinating cells of the CNS, are very sensitive to the effects of injury. Oligodendrocyte death at the site of injury occurs by apoptosis with secondary axonal demyelination [27, 28]. Glutamatergic excitotoxicity with increased oxidative stress, release of pro-inflammatory cytokines and increased extracellular ATP levels are the main causes of oligodendroyte death [29]. Since mature oligodendrocytes are the source of myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) [30], loss of these myelin proteins follow oligodendrocyte damage [27, 31, 32]. Since surviving oligodendrocytes cannot divide, remyelination derives from recruitment of an endogenous population of oligodendrocyte precursor cells (OPC) expressing the NG2 proteoglycan [29]. Regulation of the oligodendrocyte pathway is multifactorial, and the

transcription factors Olig1, Olig2 and Nkx2.2 play specific roles during OPC differentiation and remyelination [33-36].

The promyelinating effects of progesterone have been shown in cultures of mixed glial cells and organotypic slice cultures of cerebellum [10], after toxin-induced lesions of the cerebellar peduncle of aging rats [37], during *in vitro* proliferation and differentiation of OPC [38] and after demyelination caused by experimental autoimmune encephalomyelitis, cuprizone or lysolecithin injection [39-41]. The spinal cord transection model sparks a moderate increase of NG2 + cells in the gray and white matter plus a reduction of the mRNA and protein expression of MBP, indicative of demyelination. In agreement with literature reports and our own data [42-44], the post-injury recruitment of NG2+ cells is observed in dorsal, lateral and ventral tracts of the white matter at short (3 days) and more prolonged (21 days) periods post-injury. However, this recruitment does not prevent demyelination, because immunostaining for the myelin proteins MBP and PLP, their mRNAs as well as the number of mature oligodendrocytes (i.e. CC1 + cells) are still depleted at both time periods. A depletion of transcription factors involved in myelination and oligodendrocyte maturation accompanies the myelination failure. After spinal cord injury, progesterone treatment strongly favours remyelination. We have observed that 3 days of progesterone treatment increases the recruitment of OPC and increases the mRNA levels of the myelin transcription factors Olig2 and Nkx2.2. The latter has consensus binding sites on the PLP and MBP promoters, allowing a direct control of the synthesis of the major myelin proteins [45, 46]. Therefore, progesterone´s stimulation of Olig2 and Nkx.2.2 in the progenitors should induce their differentiation into mature forms [38, 44]. Is gene transcription involved in these progesterone effects? While a PR/ glucocorticoid receptor (GR) hormone-response-element is present in a few gene promoters, it is absent from others. In the case of the MBP gene, its promoter has a GR consensus element [47]. Several glucocorticoid-responding genes share the same bases in DNA with the PR response element [48, 49]. However, the GR / PR consensus sequence is absent from other progesterone-responding genes, suggesting that indirect or alternative mechanisms may operate for progesterone effects on these molecules.

Additionally, the changing pattern of oligodendrogenesis obtained after 21 days of progesterone treatment again suggests a myelination drive. At this time period, the early rise of OPC promoted by early progesterone treatment subsided, with a concomitant increase of mature oligodendrocytes, suggesting a progesterone-induced differentiation. This possibility is ascertained after colocalization studies, showing that the proliferation marker bromodeoxyouridine (BrdU) colocalizes with mature oligodendrocytes bearing the CC1 marker. These changes are accompanied by increased expression of PLP mRNA and protein and by increased mRNA of the transcription factor Olig1. Olig1 is required for oligodendrogenesis and for repairing demyelinated lesions during demyelination caused by cuprizone or lysolecithin application [50], and the same reasoning could be applied to spinal cord injured rats. In conclusion, these experiments strongly support the hypothesis that progesterone may promote a myelination drive favouring differentiation of proliferating progenitors into mature, myelinating oligodendrocyte forms in the injured tissue.

As opposed to damage inflicted by spinal cord injury to neurons and oligodendrocytes, functional and morphological alterations of astrocytes and microglial cells suggest an increased reactivity. Astrocytes show hypertrophic and proliferative changes [51], with a peak activation within 3 days after injury [52]. Reactive astrocytes change their gene expression, release proinflammatory mediators that attract macrophages and microglia and induce their local and distal proliferation. Astrocyte reactivity might result from specific signaling cascades targeting microglia and other inflammatory cells [53]. However, the significance of astrogliosis for the recovery of spinal cord function is controversial [54, 55]. Spinal cord injury also inflicts a strong inflammatory reaction characterized by microglial activation and infiltration of neutrophils, monocytes/macrophages and dendritic cells [56]. Activated immune cells release proinflammatory cytokines, reactive oxygen species and toxic levels of nitric oxide, damaging neurons and axons and causing demyelination [57, 58]. Thus, overshadowing the immune response with progesterone may be a useful strategy to avoid secondary damage to the lesioned spinal cord, as has already been shown for traumatic brain injury [59-61]. We have demonstrated that after spinal cord injury, progesterone attenuates the reactive microgliosis, inhibiting microglia proliferation and the release of several pro-inflammatory cytokines [62].

In conclusion, spinal cord injury and progesterone modify the distribution of glial cell populations in opposite directions (Fig. **1**). After spinal cord injury, astrocytes and microglial cells increase from 9% to 23% and from 9% to 26%, respectively, compared with sham-operated rats. Simultaneously, mature oligodendrocytes decrease from 72% to 20% (Fig. **1**). Progesterone, instead, creates a pro-myelinating environment by changing the glial cell distribution, since 60% of the total cells counted are oligodendrocytes whereas astrocytes and microglia account for only 9% and 14%, respectively (Fig. **1**). Within this cellular and molecular context progesterone emerges as a cytoprotective molecule for oligodendrocytes and its progenitors, owing its effects at least in part to the inhibition of the inflammatory and harmful interplay between astrocytes and microglia. On the other hand, the multiplicity of effects that progesterone and reduced derivatives exert on OPC proliferation / differentiation and on myelin genes make us consider that progesterone targets the oligodendrocyte lineage. Whereas there is no current evidence that progesterone prevents apoptotic death of mature oligodendrocytes, it may increase survival of oligodendrocyte progenitors due to its anti-inflammatory properties. Thus, animal models of spinal cord injury further support the role of progesterone as a promising therapeutic agent for humans with spinal cord injury.

PROGESTERONE EFFECTS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE), A MODEL FOR MULTIPLE SCLEROSIS

EAE is an accepted model for human multiple sclerosis (MS), a common demyelinating disease of autoimmune

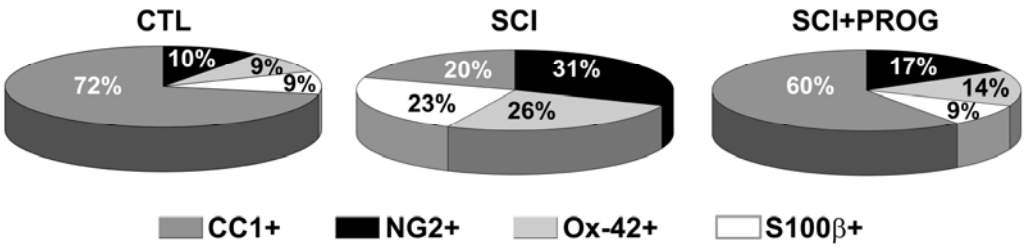

Fig. (1). Schematic distribution of glial cell populations after spinal cord injury (SCI) and progesterone (PROG) treatment for 21 days. Data are presented as % of each immunopositive cell group over the total number of cells counted in 0.5mm^2 of the spinal cord. Group labelling: CTL (intact animals), SCI (injured spinal cords), SCI+PROG (steroid-treated rats). Microglial (Ox-42+) astrocytes (S100β+) and NG2 cells are the most abundant cell populations in the injured spinal cord. However progesterone treatment changed the distribution of glial cells, since abundance of astrocytes and microglia cells after SCI was replaced by CC1+cells. Thus, the most abundant glial type in rats with SCI receiving progesterone was the mature oligodendrocyte (CC1+ cells). Data reproduced from Labombarda *et al.* [62] with permission from Elsevier.

origin that targets the spinal cord and some regions of the brain [63, 64]. There is also strong evidence for neurodegeneration in MS, based on the high frequency of neuronal abnormalities and damaged axons [65]. Matute and Perez-Cerdá [66] have implied intrinsic defects of the oligodendrocytes, with release of myelin proteins and debris that provoke the reaction of the immune system.

As far as 80% of MS cases present a relapsing-remitting course. A significant decline in the rate of MS relapse occurs during the third trimester of pregnancy, whereas a significant increase develops during the 3 months postpartum. The pregnancy effect may be due to the high levels of sex steroids circulating in pregnancy, whereas post-partum relapses correlate with decreased levels of circulating steroids [67-71]. Therefore, the potential therapeutic benefit of sex steroid hormones for MS patients has been taken into consideration. Leitner [72] has suggested that MS demyelination may be due to the lack of brain neurosteroids. In support of this hypothesis, Noorbakhsh *et al.* have reported that the brain of MS patients and EAE rodents present reduced levels of neurosteroids [68].

We have induced EAE in C57Bl6 female mice using a myelin oligodendrocyte glycoprotein peptide (MOG_{40-54}) [34, 40, 73]. One week before EAE induction, mice receive single pellets of progesterone weighing either 20 or 100 mg or remain free of steroid treatment. On average, mice develop clinical signs of EAE about 10 days following MOG administration. Clinical correlates of spinal cord pathology include loss of tail tonicity, rear limb paralysis and even death [39, 40, 74]. A number of treatments have been used to prevent the development or halt the progression of EAE, including sex steroids [34, 74, 75]. We have studied whether progesterone pretreatment influences the clinical aspect, and attenuates neuropathology and neurochemical abnormalities of the spinal cord of EAE mice. The spinal cord white matter of EAE mice shows inflammatory cell infiltration and circumscribed demyelinating areas, demonstrated by reductions of luxol fast blue (LFB) staining, of MBP and PLP immunoreactivity (IR) and PLP mRNA expression. In motoneurons, mice with EAE show a reduced expression of the alpha 3 subunit of Na, K-ATPase mRNA [39, 74]. In contrast, EAE mice receiving progesterone show less inflammatory cell infiltration, recovery of myelin proteins and Na, K-ATPase mRNA levels. Clinically, progesterone produces a moderate delay in disease onset and reduces the clinical scores. In this way, exogenously administered progesterone attenuates disease severity, and reduces the inflammatory response and the occurrence of demyelination in the spinal cord during the acute phase of EAE [39]. However, endogenously produced steroids (neurosteroids) may also play a significant role during the course of EAE. Work by Melcangi and coworkers [76] has shown that in rats with induced EAE, there are dimorphic changes in the levels of progesterone and derivatives in different CNS 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.

It has been shown that axons are not spared in EAE [77]. In a separate study, we have evaluated if progesterone also protects axons from immune attack [78]. On day 16th after EAE induction, we have determined in EAE mice with or without progesterone pretreatment and in control mice several axonal parameters, such as: axonal density in semithin sections of the spinal cord ventral funiculus; presence of amyloid precursor protein (APP) immunopositive spheroids as an index of damaged axons; levels of the growth associated protein GAP43 mRNA and immunopositive cell bodies, as an index of aberrant axonal sprouting. Steroid-naive EAE mice have shown decreased axonal density, shrunken axons, abundance of irregular vesicular structures, degenerating APP+ axons, increased expression of GAP43 mRNA and immunoreactive protein in motoneurons. Instead, in EAE mice receiving progesterone treatment we have observed increased axonal counts, high proportion of small diameter axons, reduced APP+ profiles, and decreased GAP43 expression. In conclusion, progesterone protects development of axonal pathology in EAE mice. In addition to our own work, the protective effects of progesterone in the EAE model have been recently supported by two different groups working in the USA and China [79, 80]. A summary of our work on progesterone effects in the EAE model is shown in Fig. (**2**).

So far, we have described the beneficial effects in the spinal cord of progesterone treatment in EAE mice, according to enhanced clinical, myelin and neuronal-related parameters. 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 demyelination induced by the intraspinal

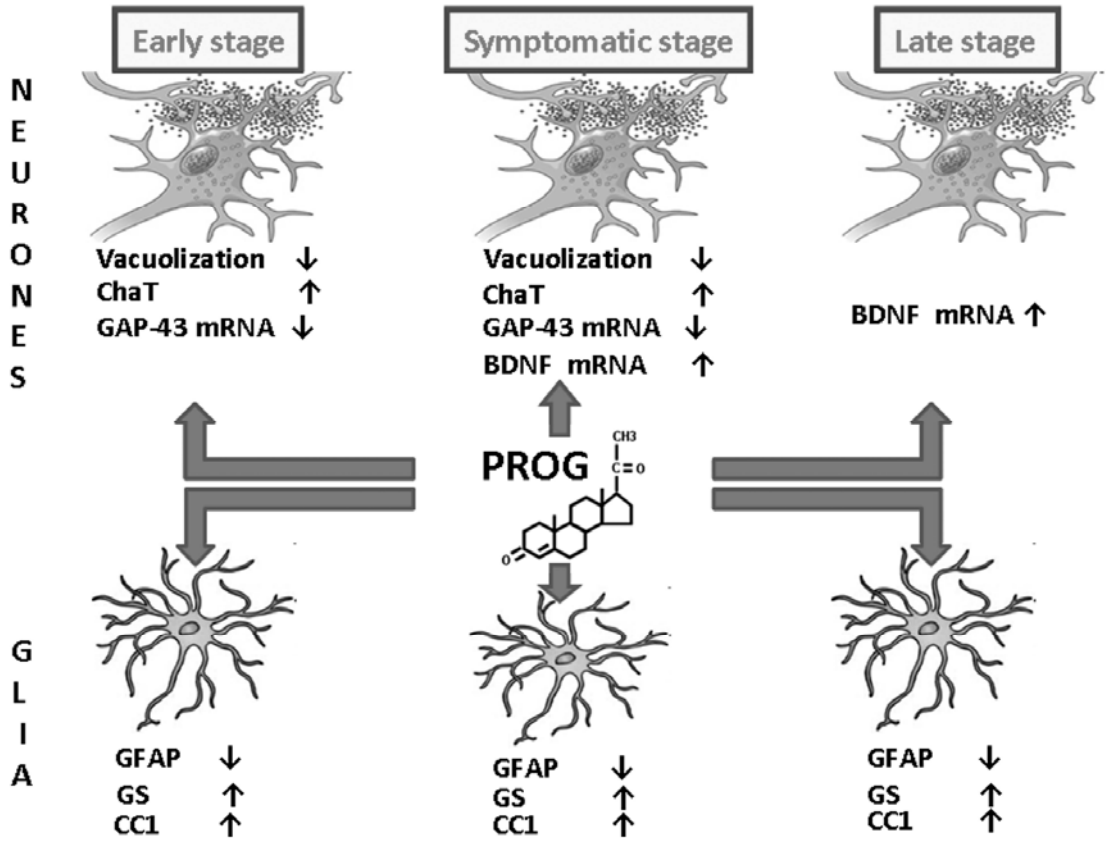

Fig. (2). Effects of progesterone on neuronal and glial cells in Wobbler mice at early progressive (1-2 months), established (5-8 months) or late stages (12 months). Progesterone increases ChAT (choline acetyltransferase) and BDNF (brain-derived neurotrophic factor) and decreases vacuolation and GAP-43 (growth-associated protein) at the established stage. Vacuolization, ChAT and GAP-43 mRNA but not BDNF responded to progesterone at early stage, whereas only BDNF mRNA responded at the late stage. In glial cells, progesterone decreases GFAP (glial fibrillary acidic protein) in astrocytes and increases CC1+ cells (mature oligodendrocytes), glutamine synthase (GS in undetermined cells) and also BDNF + oligodendrocytes in the established stage. GFAP, GS and CC1 but not BDNF responded to progesterone at the early and late stages. Data modified from Meyer, M., PhD Thesis, Faculty of Medicine, University of Buenos Aires, 2012.

injection of lysophospatidylcholine (LPC) [40]. In this study, C57Bl6 adult male mice remained untreated or received a single 100 mg progesterone implant, which increased circulating steroid levels comparable to those of mouse pregnancy. Seven days afterwards, mice received a single injection of LPC into the dorsal funiculus of the spinal cord. A week after, myelin status was investigated by histochemical staining for total myelin (Luxol Fast Blue), MBP immunohistochemistry, the density of oligodendrocyte progenitors (NG2+ cells) and mature oligodendrocytes based on CC1 staining. Microglial reactivity was investigated using immunostaining for the microglial / macrophage marker OX-42+ and for the microglial marker CD11b mRNA using real-time PCR. Results have shown that progesterone pretreatment of LPC-injected mice decreased by 50% the area of demyelination, evaluated by either LFB staining or MBP immunostaining, increased the density of NG2+ cells and of mature CC1+ oligodendrocytes and decreased the number of OX-42+ cells and CD11b expression respect of steroid-untreated LPC mice.

These results suggested that in focal demyelination model progesterone behaves as a promyelinating, anti-inflammatory factor similarly to results obtained in EAE mice. The LPC model indicates that progesterone produces promyelinating and anti-inflammatory effects at the spinal cord level. Therefore, dual progesterone effects may take place in EAE: first, peripheral effects by prevention of immune system attack on the spinal cord, and second direct anti-inflammatory and promyelinating actions in the spinal cord. These possibilities expand the range of progesterone activities. Recent theories of MS consider the disease being primarily inflammatory and then neurodegenerative, whereas others put the accent on neurodegeneration [66, 81]. It is hoped that progesterone could exert similar effects on MS patients, favoring remyelination.

PROGESTERONE PROTECTION IN SPINAL CORD NEURODEGENERATION

The Wobbler mouse mutant is useful to study progesterone effects in a motoneuron degeneration model. Wobblers suffer a mutation in the gene coding for Vps_{54} (vacuolar vesicular protein sorting) present in chromosome 11. Wobblers are one of the accepted models for the sporadic form of amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy [82, 83].

Motoneurons of Wobbler mice undergo a dramatic perikaryal vacuolar degeneration similar to paraptosis [84-

86], accompanied by astrocytosis [87, 88] and microglial activation [82]. Wobbler astrocytes show reduced capacity to take up glutamate, a pathological condition leading to neuronal damage [83]. Mitochondrial morphological abnormalities of motoneurons correlate with decreased oxygen consumption and respiratory complex activity [89, 90]. The clinical, biochemical and morphological improvement caused by treatment with antioxidants, antiglutamatergic drugs, steroids, nitric oxide inhibitors and BDNF [84, 91-93] strongly support a role of reactive oxygen species in Wobbler pathology. Abnormal expression of several genes has been reported in the Wobbler. These include decreased expression of Na, K-ATPase, an enzyme maintaining ion homeostasis, membrane potential and cell excitability, BDNF, a trophic and promyelinating growth factor, ChAT, necessary for neuromuscular contraction, glutamine synthase (GS), which prevents excess glutamate accumulation, and hyperexpression of the GAP43, a cause of aberrant synaptogenesis [85, 94]. Deficits also exist in retrograde axonal transport [85, 94]. On the other hand, Wobblers show increased expression and/or activity of nitric oxide synthase (NOS). Neurotoxic levels of NO become a noxious factor for mitochondrial respiratory chain complexes [95]. Wobbler mice live shortly and the disease is accompanied by muscle atrophy and reduced muscle strength [85].

To study progesterone effects on the degenerating spinal cord of Wobblers we have employed symptomatic mice showing full-blown clinical expression of the disease, i.e., ambulatory difficulties, muscle atrophy and forelimb flexion [96]. These mice remain untreated or receive progesterone during 18 days. Progesterone has successfully counteracted most neurochemical abnormalities. Thus, vacuolated motoneurons are reduced 6-fold, with a concomitant reduction of NOS active neurons. Electron microscopy observations have demonstrated a considerable reduction of disrupted mitochondrial membranes. Longer exposure of Wobbler mice to progesterone for 60 days has also increased neuronal BDNF mRNA, ChAT immunoreactive motoneurons and Na, K-ATPase mRNA levels in the spinal cord [94]. Progesterone attenuates the ongoing atrophy of Wobbler mice forelimb biceps bracchii, implying an enhanced neuromuscular function [96]. Thus, restoration of BDNF expression following long-term exposure to progesterone may be essential for the ailing motoneurons, and we have hypothesized that BDNF could be an intermediate of progesterone effects in the degenerating spinal cord. BDNF binds to its cognate receptor TrkB which leads to trophic and beneficial effects, or to the pan neurotrophic receptor $p75^{ntr}$, which mediates death signals [97]. Although progesterone effects on TrkB are largely negative, we have observed a down-regulation of the expression of $p75^{ntr}$ (Gonzalez Deniselle *et al.* unpublished results). Thus, progesterone could play a dual role, in part by stimulation of endogenous BDNF, and in part by decreasing its binding to the $p75^{ntr}$ death receptor.

We have additionally investigated the response of the Wobbler mouse to progesterone treatment at different time periods of the disease [98, 99]. To this end, genotyped (wr/wr) Wobbler mice have been divided into three periods: early progressive (1-2 months), established (5-8 months) or late stages (12 months). As controls, we have used age-matched wild-type animals (NFR/NFR). One half of each group then received progesterone for 18 days. In these studies, we have evaluated separately effects on motoneurons and glial cells. In motoneurons, we have shown that progesterone therapy significantly reduced motoneuron vacuolation, enhanced ChAT immunoreactive perikarya and reduced the aberrant expression of GAP43 during the early progressive and established stages. Although BDNF depletion occurs in the untreated Wobblers at the three stage periods, progesterone restores BDNF expression in motoneurons at the established and late stages only. At all stage periods, untreated Wobblers present high density of GFAP+ astrocytes and decreased number of GS and CC1+ mature oligodendrocytes. Progesterone treatment normalizes the density of GFAP+ astrocytes and up-regulates GS+ and CC1+ cell number. These data has reinforced the view that therapeutic benefit seems stage-dependent and varies depending on the target analyzed [98, 99].

A further study of our group has analyzed progesterone effects on mitochondrial-associated parameters of symptomatic Wobbler mice. The activities of mitochondrial respiratory chain complexes I, II-III and IV and protein levels of mitochondrial and cytosolic NOS have been determined in cervical and lumbar cords from control, Wobbler and Wobbler mice receiving a progesterone implant for 18 days. We have found a significant reduction of complex I and II-III activities in mitochondria and increased protein levels of mitochondrial, but not the cytosolic form of NOS (nNOS), in the cervical cord of Wobbler mice. Progesterone treatment prevents the reduction of cervical region complex I and the increased level of mitochondrial nNOS. Wobbler motoneurons also show accumulation of amyloid precursor protein (APP) immunoreactivity and decreased activity and immunostaining of manganese - dependent superoxide dismutase (MnSOD) [100]. Progesterone treatment avoids these abnormalities. Therefore, administration of progesterone to clinically afflicted Wobblers (1) prevents the abnormal increase of mitochondrial nNOS and normalizes respiratory complex I; (2) decreases APP accumulation, a sign of axonal degeneration, and (3) increases superoxide dismutation. Thus, progesterone prevents the pronounced mitochondriopathy developing in motoneurons from the Wobbler mouse cervical spinal cord.

What lessons can be learned from this animal model? An important one is that neurodegeneration is not an irreversible phenomenon. We envision that the protective effects of progesterone demonstrated in the Wobbler mouse might provide useful information about novel therapeutic approaches for patients suffering from diseases targeting motoneurons.

PROGESTERONE AND NEUROPATHIC PAIN

A growing amount of literature supports the notion that neuroactive steroids display key regulatory effects in the control of pain and might represent a potential therapeutic approach for the treatment of chronic pain [101, 102]. Progesterone, in particular, mediates gestational antinociception [103], contributes to sex-related differences in pain [103], reduces pain sensitivity in intact rats [104] and attenuates neuropathic pain-associated behaviors in animals

displaying a peripheral nerve injury [105] or diabetic neuropathy [106]. Recent reports from our laboratory show that progesterone administration to animals with either peripheral or CNS injuries, inhibits the development of neuropathic pain behaviors, and prevents the maladaptive expression of several pain-related molecules with crucial roles in spinal nociceptive processing [107, 108].

Neuropathic pain develops after a lesion or disease of the somatosensory system and is a major concern for patients with spinal cord injury, with an estimated incidence that ranges from 40 to 60% [109]. These patients, already burdened with the disability of paralysis, emotional trauma and spasticity, must contend with severe unrelenting pain. This chronic pain condition is characterized by the presence of both spontaneous and induced pain. Allodynia, pain elicited by normally innocuous stimuli, is the most frequent neuropathic-pain associated behavior both in humans and animals. Unfortunately, this maladaptive and unrelenting pain still remains extremely difficult to treat [109]. Currently available pharmacotherapy has limited efficacy and adverse side effects.

Although the precise mechanisms underlying neuropathic pain remain elusive, several maladaptive molecular events are known to contribute to the observed pain-related behaviors following injury [108]. In this regard, both the increased expression and/or the activity of the NMDA receptors play a critical role in the development and maintenance of chronic pain. The functional NMDA receptor contains an obligatory NR1 subunit in combination with at least one of the four NR2 subunit family members, of which NR2A and NR2B are the most abundant in the dorsal horn. Increased phosphorylation of the NR1 subunit correlates with the presence of neuropathic pain behaviors. Furthermore, the gamma isoform of the protein kinase C (PKCγ), has been shown to amplify the NMDA receptor mediated-circuit by inducing the phosphorylation of NR1 [110]. Dynorphin, an endogenous opioid peptide, has been originally identified as a ligand for the kappa opioid receptor (KOR) with analgesic properties, but later on it has been associated with the maintenance of neuropathic pain [103]. This switch from antinociceptive to pronociceptive properties has been related to the ability to bind to the NR1 subunit through a non-opioid mediated mechanism that in turn activates the NMDA receptor [103].

We have recently shown that after spinal cord injury there is a significant increase in the expression of NMDA receptor subunits NR1, NR2A and NR2B in the dorsal spinal cord [107]. We have also detected increased mRNA levels corresponding to PKCγ and pre-pro-dynorphin (ppD), as well as basal levels of expression of KOR [107, 108]. The number of neuronal profiles exhibiting PKCγ, NR1 or pNR1 immunoreactivity is also increased in the dorsal horn of injured animals. Interestingly, progesterone administration prevents the injury-induced increase in NMDA receptor subunits and PKCγ mRNA levels and immunoreactive profiles, does not modify the elevated ppD mRNA levels, and results in increased KOR expression [104]. In accordance with the proposed role of these molecules in the dorsal horn pain circuit, progesterone has also prevented the development of neuropathic pain-associated allodynia [107]. Thus, the behavioral and molecular changes taking place during progesterone administration suggest that this steroid could be favoring a scenario that inhibits and/or attenuates the onset of neuropathic pain after experimental spinal cord injury.

The pathophysiology of neuropathic pain after spinal cord injury involves multiple factors such as the imbalance of excitatory and inhibitory neurotransmission, an exacerbated inflammatory response and cell death [108]. Therefore, drugs used for the treatment of chronic pain should be able to block the multiple cellular and molecular events leading to damage, at the different control levels. In this context, progesterone is an excellent candidate since, after spinal or brain injury, it exerts concerted beneficial influences on multiple processes, as already discussed earlier in this review [3, 4]. Thus, progesterone may be contributing to limit the spread of pathological changes in the injured spinal cord, avoiding the onset of pain.

It still remains unclear whether the response elements in the promoters of the genes encoding the molecules involved in nociception allow a direct control of their expression through the PR. However, multiple signals have been involved in the regulation of PKC and the NMDA receptor subunits genes even in the absence of steroid-response elements. Thus, the possibility exists that their transcription may be regulated through the cross-talk of PR with members of the AP-1, NFκB and Sp-family of transcription factors [111] or through its interactions with the Src/Ras/MAPK and the c-AMP signaling pathways [112].

Other mechanisms could not be precluded when considering progesterone analgesic effects, including PGRMC1 [13] and the sigma-1 receptor, which is strongly expressed in the dorsal spinal cord and is associated with central sensitization and pain [113]. Furthermore, other steroid mediated actions derive from the rapid conversion of progesterone into DHP and allopregnanolone in the dorsal spinal cord [114]. These reduced metabolites, acting as positive allosteric modulators of $GABA_A$ receptor complex [7, 23], or by enhancing specific $GABA_A$ receptor subunits [115], may play an important role in mediating the reduction of allodynic behaviors observed after progesterone administration.

The role of allopregnanolone, endogenously synthesized within the dorsal spinal cord, in modulating neuropathic pain has indeed been extensively studied. Thus, expression and activity of enzymes necessary for the synthesis of allopregnanolone, in particular the 3α-hydroxysteroid dehydrogenase, are upregulated in the dorsal horns in response to experimentally induced neuropathic pain. Functional studies revealed that the intrathecal administration of allopregnanolone induced analgesia in neuropathic-pain rats, whereas inhibition of allopregnanolone synthesis potentiated both thermal hyperalgesia and mechanical allodynia in neuropathic rats [101].

Further studies are needed in order to uncover the diverse signaling mechanisms involved in progesterone observed effects. However, the results shown here are promising and provide compelling evidence that supports the early use of progesterone to prevent the development of neuropathic pain.

PROGESTERONE PREVENTION OF ISCHEMIC STROKE

Progesterone is also a promising candidate for neuroprotective strategies after stroke, a major cause of death and neurological disability, confining one-third of stroke survivors to nursing homes or institutional setting [116]. The only approved treatment for acute stroke is thrombolysis with tissue plasminogen activator, but it can only be used in 10% of patients [117, 118]. Progesterone may represent safe and effective therapy that can be offered to a higher percentage of patients. Indeed, experimental studies have demonstrated the efficiency of progesterone treatment in reducing lesion volume and improving functional recovery following either transient or permanent occlusion of brain arteries [119-122]. Until recently, the widely accepted assumption is that neuroprotective effects of progesterone may be mainly mediated by its metabolite allopregnanolone, which does not bind to PR but to membrane γ-*aminobutyric* acid type A ($GABA_A$) receptors, the major inhibitory neurotransmitter receptors in the brain [123, 124]. There is indeed experimental evidence for a beneficial influence of allopregnanolone on neuron viability after brain injury or in neurodegenerative conditions [125-127].

In a recent study [128] we aimed to test the hypothesis that the nuclear receptors of progesterone PR may play a key role in the neuroprotective actions of progesterone in stroke. We showed that progesterone, decreased infarct volume, edema formation and improved functional outcome after stroke and that progesterone receptors PR are required for this neuroprotection. To study the role of nuclear receptor of progesterone in neuroprotection, we used the MCAO model: an experimental model of transient focal cerebral ischemia by occluding the left middle cerebral artery during 1 hour with an intraluminal filament followed by reperfusion. We used wild-type mice and PRKO mice which do not express the nuclear progesterone receptors (PR). 24 hours post MCAO, PRKO mice showed larger infarcts than wild-type mice. These results showed that PR inactivation results in increased vulnerability of the brain to ischemic damage. In addition, they pointed to a role of endogenous brain progesterone in PR-dependent neuroprotective signaling at the 24 hours after stroke. We also observed at 6hours after MCAO, that stroke induced an increase in the levels of the two neurosteroids progesterone and 5α-dihydroprogesterone, both endogenous ligands of PR. Although endogenous progesterone *via* PR did appear to protect the brain during the first 24 hours of recovery, the damage continued to evolve during the next 24 hours rendering Wild-type mice and PRKO mice equally damaged at 48h. Indeed, in contrast to the 6h and 24h time points, no influence of the *PR* genotype on infarct volume was observed at 48 hours. These results suggest an acute and transient protective effect of endogenous PR activation, albeit insufficient to overcome the deleterious effects of ischemia on delayed cell death. Longer-term neuroprotection after stroke requires additional treatment with progesterone and is also PR-dependent. Indeed, pharmacological treatment with progesterone, decreased infarct volume, edema formation and increased motor coordination. These beneficial effects of progesterone are observed in wild-type mice but not in PRKO mice (Fig. **4**). In addition, the potent and selective PR agonist Nestorone® is also effective in reducing infarct volume and improving functional outcome after stroke, confirming the key role of progesterone receptors PR. In contrast to progesterone, levels of the neurosteroid allopregnanolone, which modulates gamma-aminobutyric acid type A receptors, did not increase after stroke, but its administration protected both wild-type and PR-deficient mice against ischemic

In conclusion, our study demonstrates that PR is an essential key for early endogenous neuroprotection and it might serve as pharmacological drug target for stroke therapy and allopregnanolone may not be an endogenous neuroprotective agent. However allopregnanolone treatment can protect the brain against ischemic damage by signaling mechanisms not involving PR.

CONCLUDING REMARKS: ANIMALS MODELS AS TOOLS FOR TRANSLATIONAL MEDICINE

Results obtained in animal models of human disorders are not easily translated to the human patient. However, few exceptions make us optimistic on this matter. This is the case of traumatic brain injury (TBI), in which the neuroprotective effects of progesterone obtained in rats have recently been translated to a clinical trial, named "ProTECT" [progesterone for traumatic brain injury (TBI)]. The usefulness of intravenous progesterone as a treatment for moderate to severe TBI has been investigated. Results of this phase II trial, although involving a small number of patients, were encouraging as they suggest a reduction in the mortality rate and better functional outcomes in the progesterone-treated patients [129] These results are in agreement with another recent clinical trial involving progesterone administration [130].

ALS is another disorder where treatments tested in animal models might provide a clue to prolong patient survival. Due to its progressive and fatal outcome a marginal delay in mortality has been observed with the antiglutamatergic drug Riluzole [131]. Results of progesterone neuroprotection in the Wobbler mouse model of motoneuron degeneration, encouraged studies to examine serum progesterone levels in ALS patients. We have searched for an association between progesterone levels with prognostic factors and survival of ALS patients [132]. We have shown in a cohort of ALS patients that progesterone serum concentrations are 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 have shown lower progesterone levels in comparison to spinal onset patients, a condition of better disease prognosis. In a second study, preliminary data obtained from ALS patients have disclosed that high levels of circulating progesterone positively correlated with a reduced need of assisted respiratory ventilation (Gargiulo-Monachelli *et al*, unpublished data). Therefore, it is possible that progesterone levels in ALS patients may be a biological marker of ALS prognosis and survival.

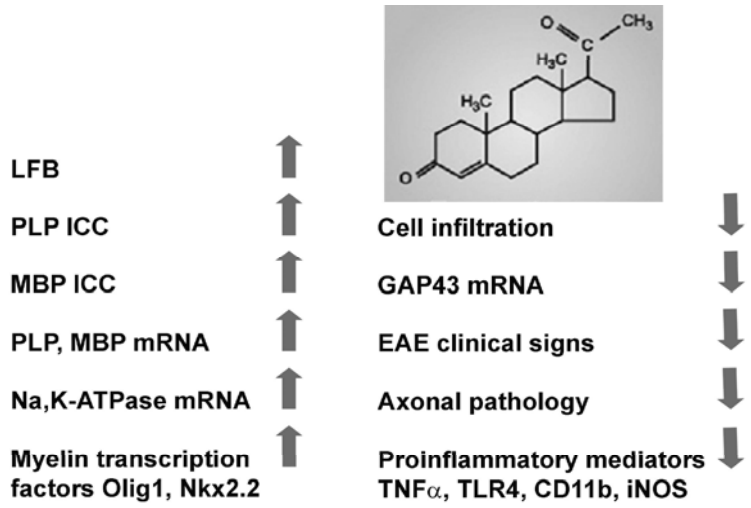

Fig. (3). Progesterone effects in experimental autoimmune encephalomyelitis. Progesterone pretreatment increases total myelin staining (LFB, luxol fast blue), protein and mRNA expression of myelin basic protein (MBP) and proteolipid protein (PLP), the Na, K-ATPase and myelin transcription factors Olig1, Olig2 and Nkx.2.2. In contrast, inflammatory cell infiltration, GAP-43 mRNA, clinical signs, axonal patholopgy and proinflammatory factors are decreased by progesterone in this animal model.

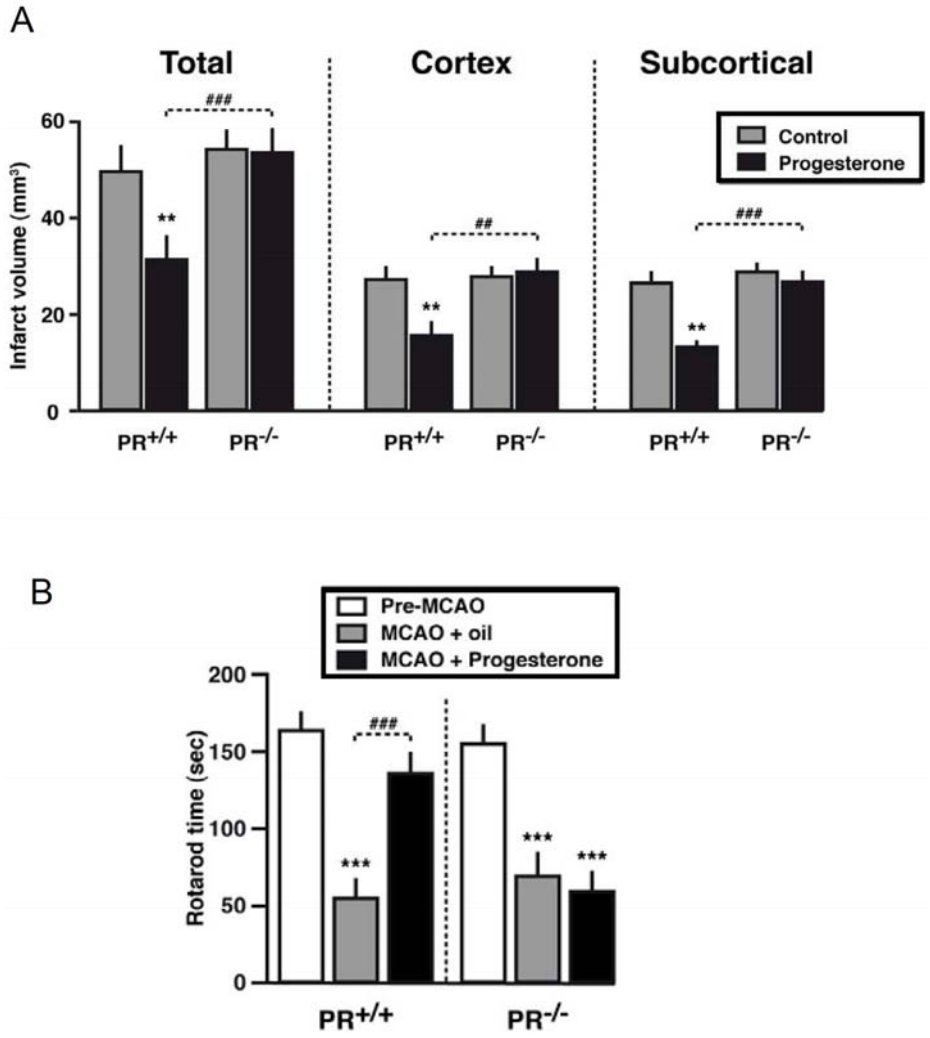

Fig. (4). Improvement of neurological outcomes at 48 h after MCAO requires treatment by progesterone and is *PR* dependent. A: Reduction in infarct volume by progesterone.**, $P < 0.01$ as compared with control mice; ###, $P < 0.001$; ##, $P < 0.01$ as indicated. B: Effects of MCAO and progesterone treatment on the time mice remained on a rotarod. ***, $P < 0.001$ compared with preischemia performance; ###, $P < 0.001$ as indicated. Data represent means ± SEM. Adapted from Liu *et al*. [128].

We have also hypothesized that progesterone effects in the EAE model may be therapeutically useful for MS patients. Based on the fact that progesterone immunosuppression during human pregnancy prevents relapses [67-71], 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. The aim of this trial is to decrease the incidence of post-partum relapses [70]. This clinical trial is supported by demonstrations that progesterone protect and /or attenuate EAE development [74, 78-80]. If successful, the POPART-MUS trial will strongly support the use of sex steroid hormones as protective factors for human MS.

ABBREVIATIONS

ALS = Amyotrophic lateral sclerosis

AP-1 = Activator protein 1

APP = Amyloid precursor protein

ATP = Adenosine triphosphate

BDNF = Brain-derived neurotrophic factor

BrdU = Bromodeoxyuridine

Ca = Calcium

c-AMP = Cyclic adenosine monophosphate

CC1 = Antibody for staining oligodendrocytes

ChAT = Choline acetyltransferase

CNS = Central nervous system

DHP = 5α-dihydroprogesterone

ERK1/2 = Extracellular regulated kinase 1 and 2

GABA = Gamma butyric acid

GAP-43 = Growth-associated protein

GR = Glucocorticoid receptor

GS = Glutamine synthase

Jak = Janus kinase

KOR = Kappa opioid receptor

MAPK = Mitogen-activated protein kinase

MBP = Myelin basic protein

MCAO = Middle carotid artery occlusion

MnSOD = Manganese superoxide dismutase

MOG = Myelin oligodendrocyte glycoprotein

mPRβ = Membrane progesterone receptor beta

mPRα = Membrane progesterone receptor alpha

mPR = Membrane progesterone receptor

Na, K-ATPase = Sodium-potassium adenosintriphosphatase

NFκB = Nuclear factor kappa B

NFR = Background strain of Wobbler mouse

NG2 = Chondroitin sulfate proteoglycan marker for oligodendrocyte precursors

Nkx2.2 = Transcription factor for oligodendrocyte maturation

NMDA = N-methyl-d-aspartate

nNOS = Neuronal nitric oxide synthase

NOS = Nitric oxide synthase

NR2 = Subunit N2 of glutamate receptor

NR2A = Subunit N2A of glutamate receptor

NR2B = Subunit N2B of glutamate receptor

Olig1 = Oligodendrocyte transcription factor 1

Olig2 = Oligodendrocyte transcription factor 2

OPC = Oligodendrocyte precursor cells

OX-42 = Marker of microglial cells

$p75^{ntr}$ = Pan neurotrophin receptor

PCR = Polymerase chain reaction

PGRMC1 = Progesterone receptor membrane component 1

PKCγ = Protein kinase C gamma

PLP = Proteolipid protein

ppD = Pre-pro-dynorphin

PR = Progesterone receptor

PRA = Progesterone receptor A

PRB = Progesterone receptor B

PRKO = Progesterone receptor knockout mice

Ras = Rat sarcoma

Sp = Specificity protein of transcription factors

STAT = Signal transducer and activator of transcription

Src = Sarcoma

TBI = Traumatic brain injury

TrkB = Tropomyocin receptor kinase B receptor

Vps_{54} = Vacuolar protein sorting gene 54

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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