Progesterone and Nestorone Promote Myelin Regeneration in Chronic Demyelinating Lesions of Corpus Callosum and Cerebral Cortex

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Multiple Sclerosis affects mainly women and consists in intermittent or chronic damages to the myelin sheaths, focal inflammation, and axonal degeneration. Current therapies are limited to immunomodulators and antiinflammatory drugs, but there is no efficient treatment for stimulating the endogenous capacity of myelin repair. Progesterone and synthetic progestins have been shown in animal models of demyelination to attenuate myelin loss, reduce clinical symptoms severity, modulate inflammatory responses and partially reverse the age-dependent decline in remyelination. Moreover, progesterone has been demonstrated to promote myelin formation in organotypic cultures of cerebellar slices. In the present study, we show that progesterone and the synthetic 19-nor-progesterone derivative Nestorone® promote the repair of severe chronic demyelinating lesions induced by feeding cuprizone to female mice for up to 12 weeks. Progesterone and Nestorone increase the density of $NG2^+$ oligodendrocyte progenitor cells and CA II^+ mature oligodendrocytes and enhance the formation of myelin basic protein (MBP)- and proteolipid protein (PLP)-immunoreactive myelin. However, while demyelination in response to cuprizone was less marked in corpus callosum than in cerebral cortex, remyelination appeared earlier in the former. The remyelinating effect of progesterone was progesterone receptor (PR)-dependent, as it was absent in PR-knockout mice. Progesterone and Nestorone also decreased (but did not suppress) neuroinflammatory responses, specifically astrocyte and microglial cell activation. Therefore, some progestogens are promising therapeutic candidates for promoting the regeneration of myelin.

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Key words: remyelination, oligodendrocytes, progestogens, cuprizone, progesterone receptor

Introduction

After the destruction of myelin sheaths, as observed in multiple sclerosis (MS), spontaneous remyelination occurs, but it is often incomplete and may even fail, resulting in chronic lesions with axonal dysfunction and subsequent neuronal loss. There is currently no therapy for promoting the regeneration of myelin, and drugs able to boost the endogenous capacity of remyelination would thus be of great therapeutic interest and complementary to available immunomodulatory and anti-inflammatory treatments.

In animal studies, a role of progesterone in myelination was first demonstrated in the peripheral nervous system after lesion of the mouse sciatic nerve as well as in explant cultures of rat dorsal root ganglia (Koenig et al., 1995). In the central nervous system (CNS), we previously observed that progesterone stimulated the myelination of axons in organotypic cultures of cerebellar slices prepared from postnatal rat pups (Ghoumari et al., 2003). Moreover, after the demyelination of already myelinated organotypic cultures by lysophosphatidylcholine (LPC), both progesterone and Nestorone, a highly

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potent and selective progesterone receptor (PR) agonist, favored myelin repair (Hussain et al., 2011).

In experimental autoimmune encephalomyelitis (EAE) models, used for preclinical studies of MS, preventive treatment with progesterone has been reported to reduce the severity of clinical symptoms, protect neurons, maintain myelin protein expression, and modulate inflammatory responses (Garay et al., 2007; Giatti et al., 2012; Yates et al., 2010). Moreover, when administered before or at the same time as toxin-induced demyelination, the systemic administration of progesterone attenuated axonal demyelination (Acs et al., 2009) and reduced microglial activation (Garay et al., 2011). Progesterone treatment has also been shown to modestly reverse the age-associated decline in remyelination (Ibanez et al., 2004), and to promote the proliferation and differentiation of oligodendrocyte progenitor cells (OPC) after traumatic spinal cord injury (Labombarda et al., 2009).

We show here that progesterone therapy stimulates the regeneration of myelin in the brain after chronic demyelination induced by feeding female mice during 12 weeks with the copper chelator cuprizone, a toxic agent for oligodendrocytes (Kipp et al., 2009; Matsushima and Morell, 2001). After such a long-term exposure to cuprizone, spontaneous myelin regeneration no longer takes place, at least for up to 9 weeks (Harsan et al., 2008; Hussain et al., 2013). However, following removal of cuprizone from the diet, a 3 week treatment with progesterone resulted in the replenishment of the corpus callosum with $NG2^+$ oligodendrocyte precursor cells (OPC), mature carbonic anhydrase type II ($CA\ II^+$) expressing oligodendrocytes and the recovery of MBP^+ myelin. Moreover, progesterone also promoted myelin repair in cortical gray matter. Therefore, progesterone exerts a therapeutic rather than preventive effect in this model.

The effects of progesterone on the nervous system involve multiple receptors and associated proteins, including the so-called "classical" intracellular PR, membrane progesterone receptors (mPR), membrane progesterone receptor component 1 (PGRMC1), and the sigma-1 receptor. Moreover, the conversion of progesterone to allopregnanolone, a potent modulator of γ-aminobutyric acid type A ($GABA_A$) receptors, plays a significant role in neuroprotection and in the regulation of myelin gene expression in peripheral nerves (Melcangi and Panzica, 2014; Schumacher et al., 2014). However, the remyelinating effect of progesterone after cuprizone-induced chronic demyelination involved PR, as it was completely abolished in PR-knockout mice ($PR^{-/-}$). Notably, systemic progesterone stimulated myelin regeneration less efficiently in heterozygous $PR^{+/-}$ mice, indicating PR haploinsuffiency, as previously shown for the neuroprotective effects of progesterone (Liu et al., 2012). Consistent with a key and rate-limiting role of PR in myelin regeneration, Nestorone also promoted remyelination *in vivo*. This potent and selective progestogen is being developed as a contraceptive and exhibits high selectivity and affinity for the PR, with no androgenic activity, but its therapeutic indications may extend to demyelinating diseases (Kumar et al., 2000; Sitruk-Ware and Nath, 2010).

Materials and Methods

Animals

Female C57Bl/6 mice were obtained from the Janvier breeding center (Le Genest St Isle, 53941 St Berthevin France). They were housed in a temperature-controlled room on a 12 hr light-dark cycle with food and water *ad libitum*. All experimental procedures were approved by the BEA (Bureau d'Expérimentation Animale) of the French National Institute for Health and Medical Research (Inserm).

A breeding colony of PRlacz mice, provided by J.P. Lydon and B.W. O'Malley (Baylor College of Medicine, Houston), was established in our animal facility. The PRlacz mice (C57BL6/129SvEv background) had been generated by inserting the lacZ reporter and neomycin resistance genes into exon 1 of the PR gene to disrupt the transcription of both PR isoforms (Ismail et al., 2002). These mice are a phenocopy of the previously described PR knockout mice (Lydon et al., 1995). Each female mouse was identified for its PR genotype, either as $PR^{-/-}$ (PR-knockout), $PR^{+/-}$ (heterozygous), or $PR^{+/+}$ (wild-type, WT).

Induction of Demyelination and Treatments

Intact 8 week old C57Bl/6 female mice were used in all experiments, except for one (and in the experiments with transgenic mice) in which mice were ovariectomized 1 week before the beginning of cuprizone intoxication. Female mice ($n = 10$ per group) were fed for 12 weeks with a powder meal containing 0.2% of the copper chelator cuprizone (Sigma Aldrich, St Louis, MO). Control animals were also fed with the same powder meal, but without cuprizone. After 12 weeks, cuprizone was removed from the diet and mice received for 3 weeks either a 20 mm subcutaneous Silastic implant containing progesterone (Sigma) or an empty implant. Nestorone was administered during 3 weeks by Alzet mini osmotic pumps (model #2004, with a release rate of 0.25μL/hr), delivering 1, 4, 6, 8, or 16 μg Nestorone per day, solubilized in 40% molecusol (2-OH-propyl-β-cyclodextrin). This synthetic 19-nor-progesterone derivative is 100 times more active than P on reproductive system end-points (Kumar et al., 2000). The high potency and selectivity of 19-nor-progesterone derivatives such as Nestorone make them suitable for sustained administration at very low doses via nonoral long-acting delivery systems (Sitruk-Ware and Nath, 2010).

Steroid Measurements in Plasma

At the time of sacrifice, blood was collected in heparinized tubes by cardiac puncture under isoflurane anesthesia, prior to the sampling of brains for qPCR analysis or perfusion with paraformaldehyde for immunochemistry. Progesterone was analyzed by gas chromatography/mass spectrometry (GC/MS) (Liu et al., 2012) and Nestorone by radioimmunoassay (Fraser et al., 2007).

Immunohistochemistry

Immediately after blood sampling, isoflurane anesthetized mice were perfused with a 4% paraformaldehyde PFA/PBS solution. Brains were rapidly dissected, postfixed with PFA for 48 hr and incubated for an additional 24 hr in a 33% sucrose solution. Hemispheres were then included into a Shandon Cryomatrix and stored at −80°C. Immunohistochemistry was performed on 12 µm sagittal sections. The following primary antibodies were used: rat monoclonal antibody against MBP (1/200; Millipore) to examine the extent of myelination; rabbit polyclonal antibody against the transcription factor Olig2 (1/500; Millipore), a marker of the oligodendroglial lineage; rabbit polyclonal antibody against NG2, a chondroitin sulfate proteoglycan marker of oligodendrocyte progenitor cells (OPC) (1/200; Millipore); rabbit antimouse carbonic anhydrase type II (CA II) antibody for the labeling of mature oligodendrocytes (1/1000, gift of Dr Said Ghandour, University of Strasbourg); rabbit polyclonal anti-GFAP antibody (1/1000; Abcam) for the staining of astrocytes and rabbit anti-Iba1 for identifying activated microglia (1/500; Wako); and mouse monoclonal antiphosphorylated neurofilament H antibody, detecting normal axons (SMI-31) (1/1000; Calbiochem).

Primary antibodies were detected with secondary goat antirat cyanine 3-labeled (1/200; Abcam) or Alexa fluor 488-labeled (1/500; Life Technologies), goat antirabbit cyanine 3-labeled (1/200; Abcam), or Atto 488-labeled (1/200; Sigma) antibodies, respectively. After a 3 × 5 min washing in PBS, cryosections were subjected (for MBP, Olig2, and CA II staining) or not (NG2, SMI-31, GFAP, and Iba1 staining) to antigen retrieval (pH 6) by boiling in a 10 mM sodium citrate buffer containing 0.05% Tween 20 at 96°C for 20 min. Slices were then washed 3 times with PBS and permeabilized during 30 min with a PBS solution containing 0.3% Triton X100, 4% IgG/protease free bovin serum albumin (BSA), followed by three consecutive washes in PBS. Sections were then incubated overnight at 4°C with the appropriate antibody, in a PBS solution containing 4% BSA and 0.1% Triton X100. At the end of the incubation with the primary antibody, sections were washed and rinsed with PBS and incubated for 2 hr at room temperature with the appropriate fluorophore-conjugated secondary antibody in a PBS solution containing 4% BSA. Slices were then washed 3 times in PBS and mounted in Fluoromount-G^{TM} mounting solution (Southern Biotech). The colocalization of nuclei, within cells of interest was performed by using DAPI staining (0.1 mg/mL) for 5 min, just after the incubation with the secondary antibody.

Images of the immunostained OPC, oligodendrocytes, myelin fibers, axons, astrocytes, and microglia were acquired using the image analyzing system Axiovision 4 (Carl Zeiss, Inc.) and were analyzed with the Image J software. For MBP and GFAP staining, results were expressed as percentage of control of the area of the body of the corpus callosum or of the whole fronto-cortical picture. For CA II, NG2, Olig2, and Iba1 staining, after measurement of the surface of the callosal body or whole fronto-cortical picture (0.3795 mm^2), cells were counted and expressed as cell number per 0.1 mm^2. For SMI-31 staining, the axon number was analyzed using a confocal Zeiss LSM 410 image analyzing system and the Image Tool software.

RT-qPCR Analysis

Total RNA was isolated from tissue blocks of corpus callosum/hippocampus using the Trizol Technique (Life Technologies, Invitrogen) and RNeasy Mini Kit 250 (Qiagen). Reverse transcription was performed using SuperScript II Reverse Transcriptase and dNTP set (Invitrogen), Random primers and RNasin (Promega), and followed by qPCR using the TaqMan Gene Expression Assays: PCR Master Mix, predesigned Taqman primers for MBP (Inventoried) and endogenous control (GAPDH), Thermo-Fast 96, and ABgene plates (Applied Biosystems, Thermofisher). Expression of genes was analyzed with the 7300 Systems SDS Software (Applied Biosystem) and MBP expression was normalized to reference gene GAPDH, whose expression was stable in different groups and in two independent experiments.

Statistical Analysis

Data are expressed as means ± standard error of the mean (S.E.M.). Statistical analysis was performed with GraphPad Prism 4.0. The significance of differences between means was evaluated by one-way or two-way ANOVA, followed by Bonferroni post-hoc tests for multiple comparisons. The levels of significance were $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Results

Progesterone Promotes Remyelination in Corpus Callosum

For the consistent analysis of myelin within the same area of the corpus callosum, we choosed to focus on its body. Indeed, this structure can be easily localized in the vicinity of the lateral ventricles. Feeding cuprizone to intact female mice for 12 weeks reduced MBP$^+$ myelin in corpus callosum by more than half. Importantly, in the absence of progesterone treatment, the extent of MBP immunostaining, the numbers of CA II$^+$ oligodendrocytes, GFAP$^+$ astrocytes and Iba1$^+$ microglial cells were identical at the end of the 12 weeks of cuprizone intoxication and 3 weeks later (data not shown). These results indicate that axonal demyelination and inflammation did not further progress during the 3 weeks following cuprizone withdrawal from the diet, and that there was no spontaneous regeneration of myelin during this period of time in the absence of treatment. These data are consistent with our previous observations (Hussain et al., 2013). However, when mice received a subcutaneous Silastic implant filled with progesterone for 3 weeks, MBP immunostaining was restored in the corpus callosum to levels comparable to those observed in the controls (Fig. 1A). Consistent with the changes in MBP immunostaining, MBP mRNA expression was significantly reduced by cuprizone in female mice treated with empty implants, whereas it was restored after 3 weeks of progesterone treatment (Fig. 1B).

Plasma levels of progesterone were analyzed by gas chromatography-mass spectrometry (GC-MS). They were low in intact females treated for 3 weeks with an empty Silastic

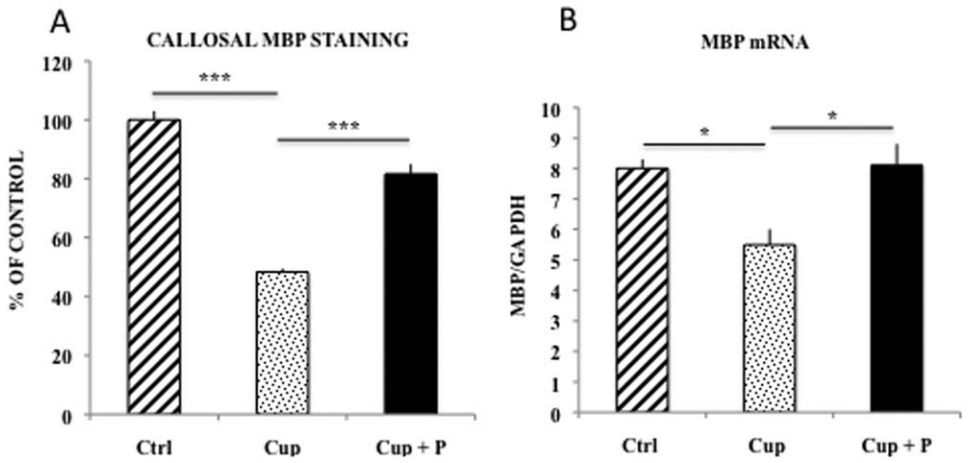

FIGURE 1: Effects of a 3 week treatment with progesterone on MBP immunostaining and mRNA expression in the body of the corpus callosum. (A) Following cuprizone intoxication during 12 weeks, intact female mice received for 3 weeks a subcutaneous Silastic implant which was empty or filled with progesterone (P). MBP immunostaining and expression were compared to cuprizone-intoxicated and further untreated mice (Cup) or to control female mice, which were not exposed to cuprizone (Ctrl). Cuprizone induced a 50% demyelination, and progesterone treatment restored MBP^+ myelin ($n = 10$ per group, corresponding to the results of two independent experiments). (B) MBP mRNA was measured in tissue blocks including corpus callosum and hippocampus (tissue blocks including corpus callosum and hippocampus). Results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) mRNA. Levels of MBP mRNA were decreased in response to cuprizone, and progesterone treatment reversed the effect ($n = 4$ per group). All data are presented as means ± S.E.M. and were analyzed by one-way ANOVA followed by Bonferroni tests. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

implant after removal of cuprizone from the diet (5.2 ± 2.2 nM). It has indeed been reported that cuprizone treatment disrupts estrous cycle in mice (Taylor et al., 2010). Treatment with Silastic progesterone implants of ovariectomized as well as intact mice induced circulating levels of the hormone similar to those observed during the diestrous phase of the cycle (45 ± 7 nM), when compared to normal females (Bastida et al., 2002; Wood et al., 2007).

While MBP^+ myelin was markedly reduced after cuprizone intoxication, the immunostaining of brain sections with an antibody against SMI-31, which reacts with phosphorylated neurofilament and labels axons of small and large calibers (Deboy et al., 2007), revealed that cuprizone did not significantly affect the density of corpus callosum axons, as observed using confocal microscopy (data not shown).

Progesterone Enhances the Number of Mature Oligodendrocytes and Their Progenitors

Remyelination of the corpus callosum in response to progesterone therapy was accompanied by the generation of new oligodendrocytes from OPC. The transcription factor Olig2, which is involved in the specification and differentiation of oligodendrocytes, is commonly used as a marker of the oligodendroglial lineage (Ligon et al., 2006; Maire et al., 2010). The number of $Olig2^+$ cells was decreased by 50% in the corpus callosum of mice receiving during 3 weeks an empty implant after cuprizone withdrawal. Treatment with progesterone during this period partially restored the density of $Olig2^+$ cells, reaching 75% of the values observed in control animals, which had not been exposed to cuprizone (Fig. 2A). Importantly, progesterone markedly increased the number of OPC expressing the transmembrane proteoglycan NG2 (Fig. 2B). As for $Olig2^+$ cells, the density of mature CA II^+ oligodendrocytes was decreased by half in response to cuprizone, but recovered to levels observed in normal mice after a 3 week treatment with progesterone (Fig. 2C). Taken together, these results indicate that progesterone promotes myelin repair in corpus callosum by stimulating the recruitment of OPC and their differentiation into myelinating oligodendrocytes.

The Remyelinating Action of Progesterone is Progesterone Receptor-Dependent

Progesterone acts on its target cells via multiple signaling pathways. To determine whether intracellular PR are involved in the remyelinating effects of the hormone, we used adult female homozygous PR-knockout mice ($PR^{-/-}$), heterozygous ($PR^{+/-}$), and wild- type ($PR^{+/+}$) mice on a C57BL6/129SvEv background (Ismail et al., 2002). Both PR isoforms are invalidated in $PR^{-/-}$ mice. Because this sub-strain showed high mortality rate during cuprizone intoxication (controls: 13%; cuprizone: 34%), mice were fed with cuprizone only during 8 weeks in this experiment, followed by a 3 week treatment with empty or progesterone-filled Silastic implants.

Exposure of these ovariectomized female mice for 8 weeks to cuprizone was sufficient for causing a 50% decrease in MBP^+ myelin in the corpus callosum. Moreover, the extent of demyelination was similar in $PR^{+/+}$, $PR^{+/-}$, and $PR^{-/-}$ mice (Fig. 3). Whereas progesterone treatment stimulated the

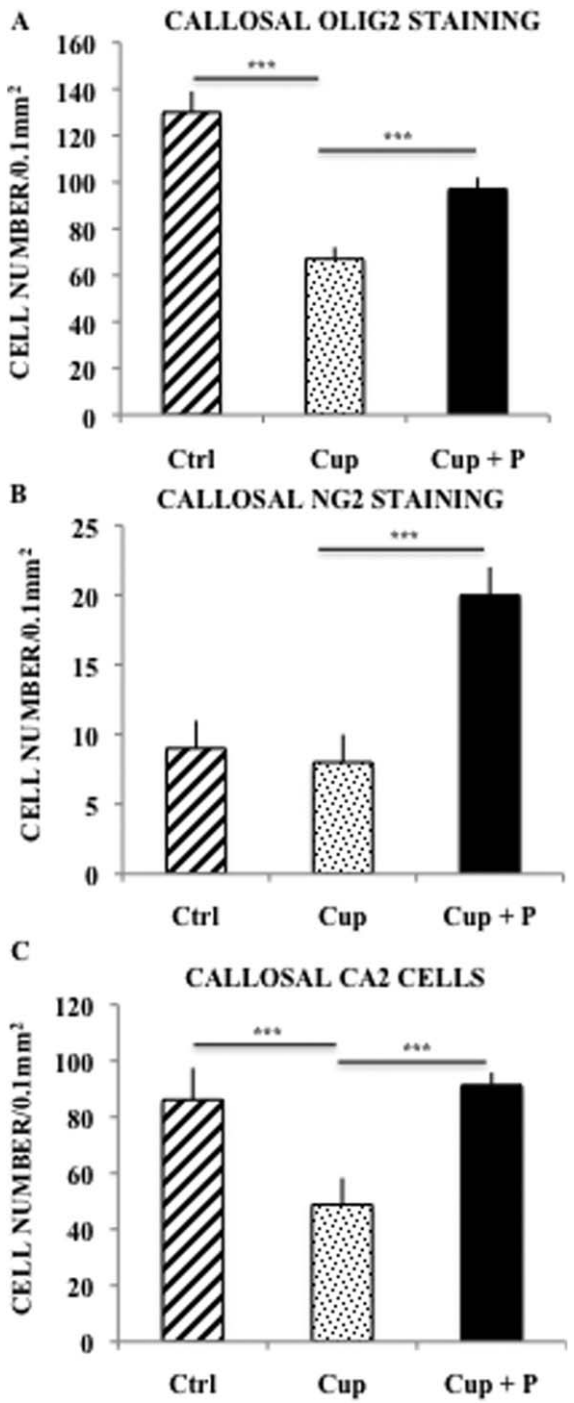

FIGURE 2: Effects of a 3 week treatment with progesterone after a 12-week cuprizone (Cup) intoxication on the number of oligodendroglial cells in the body of the corpus callosum of intact female mice. (A) Progesterone (P) enhanced the number of Olig2$^+$ cells of the oligodendroglial lineage. Results were compared to cuprizone-intoxicated/further untreated mice (Cup) or control female mice, which were not exposed to cuprizone (Ctrl) ($n = 6$ per group). (B) Progesterone increased the number of NG2$^+$ oligodendrocyte precursor cells ($n = 5$ per group). (C) Progesterone therapy also enhanced the number of mature CA II$^+$ oligodendrocytes ($n = 7$ per group). All data are presented as means ± S.E.M. and were analyzed by one-way ANOVA followed by Bonferroni tests (*$p < 0.001$).**

remyelination of corpus callosum axons in PR$^{+/+}$ mice, it was completely inefficient in PR$^{-/-}$ mice, demonstrating the requirement of PR. That PR are indeed required for the remyelinating effect of progesterone was shown by the less efficient myelin regeneration in PR$^{+/-}$ mice, lacking only one allele of the PR gene: whereas MBP$^+$ myelin reached 92 ± 8% of control values in wild-type mice treated with progesterone, it was limited to 63 ± 12% in heterozygous mice (Fig. 3). These results demonstrate an important and limiting role of PR in the remyelinating effects of progesterone.

The Synthetic 19-Norprogesterone Derivative Nestorone Also Stimulates Remyelination

The demonstration of a key role of PR in the regeneration of myelin offers new perspectives for the use of synthetic progestogens, designed to selectively target the receptors, in remyelination therapies. We thus examined the efficacy of the 19-norprogesterone derivative Nestorone in promoting myelin repair. Nestorone is indeed a highly PR-selective progestin and has about 100 times more progestational activity than natural progesterone (Kumar et al., 2000; Sitruk-Ware and Nath, 2010).

After removal of cuprizone from the diet, we administered different doses of Nestorone to intact mice for 3 weeks via subcutaneous mini-osmotic pumps (1, 4, 6, 8, or 16 μg/day). Whereas the doses of 1 or 16 μg/day failed to promote remyelination of the corpus callosum, 6 or 8 μg/day increased the number of CA II$^+$ oligodendrocytes to control levels, with the same efficacy as natural progesterone (Fig. 4). Nestorone also promoted the recruitment of NG2$^+$ oligodendrocyte precursor cells after cuprizone-induced demyelination (data not shown). Moreover, Nestorone therapy restored MBP immunostaining in corpus callosum (Fig. 5). The effects of 6 or 8 μg/day of Nestorone were comparable to those of the subcutaneous Silastic progesterone implant. The administration of 6 or 8 μg/day of Nestorone induced similar plasma levels of the progestogen (4.9 ± 0.5 nM), which were 9 times less than those of progesterone induced by the Silastic implants (45 ± 7 nM). This is consistent with the fact that Nestorone is much more potent than progesterone at the PR level.

In addition to MBP staining, we also used an antibody directed against proteolipid protein (PLP), a constitutive protein of the central nervous system myelin. Results corroborate those obtained with MBP (Fig. 7A).

Effect of Progesterone on Myelin Regeneration in Cerebral Cortex

In addition to white matter damage, a considerable amount of cortical demyelination occurs during demyelinating diseases such as MS (Geurts et al., 2012). Remarkably, demyelinating lesions in cerebral cortex have been related to the severity of physical and cognitive impairment (Calabrese et al., 2010).

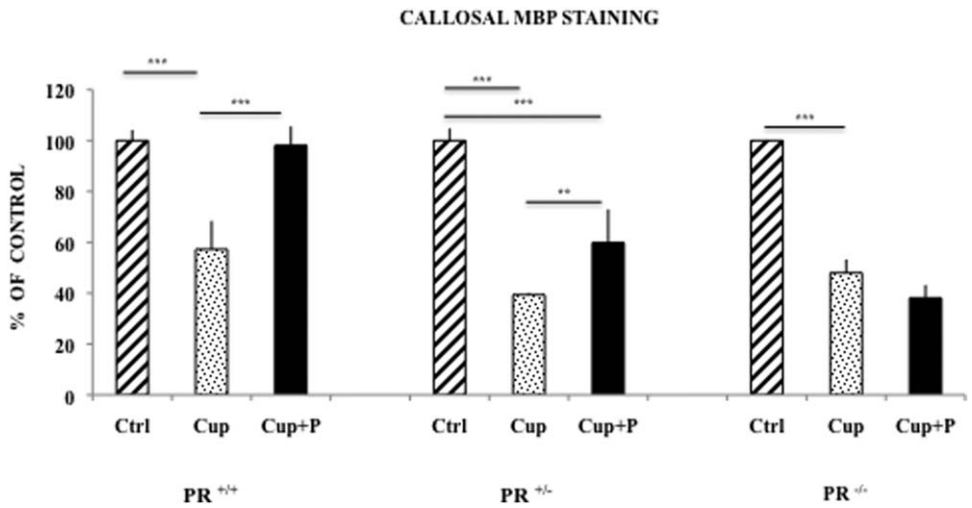

FIGURE 3: The intracellular progesterone receptor (PR) is necessary for the remyelinating effect of progesterone. Whereas treatment during 3 weeks with progesterone (P) was sufficient to restore MBP^+ myelin within the corpus callosum of castrated wild-type mice ($PR^{+/+}$) after 12 weeks of cuprizone (Cup), no remyelination was observed in ovariectomized PR-knockout mice ($PR^{-/-}$). Remyelination was only partial in castrated heterozygous mice ($PR^{+/-}$). Results were compared to cuprizone-intoxicated/further untreated mice (Cup) or control female mice, which were not exposed to cuprizone (Ctrl) ($n = 5$ per group). All data are presented as means ± S.E.M. and were analyzed by two-way ANOVA (genotype x treatment) followed by Bonferroni tests (*$p < 0.01$, ***$p < 0.001$).

Feeding mice with cuprizone also causes severe demyelination in cerebral cortex, and the model has revealed itself an excellent tool for investigating cortical demyelination and remyelination (Skripuletz et al., 2008).

Feeding cuprizone to ovariectomized female mice for 12 weeks resulted in a marked decrease in the amount of cortical MBP^+ myelin. Thus, chronic cuprizone-induced demyelination was more extensive in the cerebral cortex (about 80%) than in the corpus callosum (about 50%). Interestingly, although a 3 week treatment with progesterone significantly increased the number of CA II^+ oligodendrocytes within the cerebral cortex (Fig. 6A), it was not sufficient to significantly enhance cortical MBP staining (Fig. 6B) or PLP staining (Fig.7B). Only after 6 weeks of progesterone therapy, MBP^+ immunoreactive myelin started to recover in the cortical grey matter (Fig. 6C). Therefore, 12 weeks of cuprizone feeding to female mice induced a larger percentage of demyelination in cortical grey matter when compared to callosal white matter and myelin recovery in response to progesterone and Nestorone therapy was delayed in the cerebral cortex as compared with the corpus callosum.

Progesterone Promotes Myelin Regeneration in Ovariectomized Female Mice

Although cuprizone treatment disrupts estrous cyclicity in mice, ovarian functions progressively recover 3–4 weeks following cuprizone removal from the diet (Taylor et al., 2010). Therefore, the remyelinating effect of progesterone observed in intact female mice may be dependent on the presence of ovarian estradiol. It has indeed been reported that combined treatment with estradiol and progesterone protects more efficiently against corpus callosum demyelination by cuprizone than the individual administration of either hormone in male mice (Acs et al., 2009).

The demyelination of corpus callosum and cerebral cortex after 12 weeks of cuprizone, evaluated by MBP immunostaining, was comparable between intact and ovariectomized females (Fig. 8A). Likewise, the remyelinating effect of progesterone in corpus callosum was not dependent on the presence of the ovaries. Consistent with the results of the previous experiments in intact mice, a 3 week progesterone treatment of castrated mice was not sufficient for myelin regeneration in the cerebral cortex (Fig 8B).

Progesterone and Nestorone Attenuate Inflammatory Responses

Demyelinating lesions in the CNS are associated with the local activation and proliferation of astrocytes and microglial cells. These neuroinflammatory responses are also part of the neuropathological alterations in multiple sclerosis. We thus examined the influence of cuprizone, progesterone, and Nestorone on astrocytes and microglial cells.

Within the corpus callosum and cerebral cortex, cuprizone-induced demyelination resulted in strong increase in reactive astrocytes, as revealed by a marked increase in glial fibrillary acidic protein (GFAP) immunolabelling (Figs. 9A and 9B). In both brain structures, treatment with progesterone or Nestorone (8 μg/day by minipump) during 3 weeks after cuprizone removal exerted a significant inhibitory effect (but not a complete suppression) on GFAP immunoreactivity.

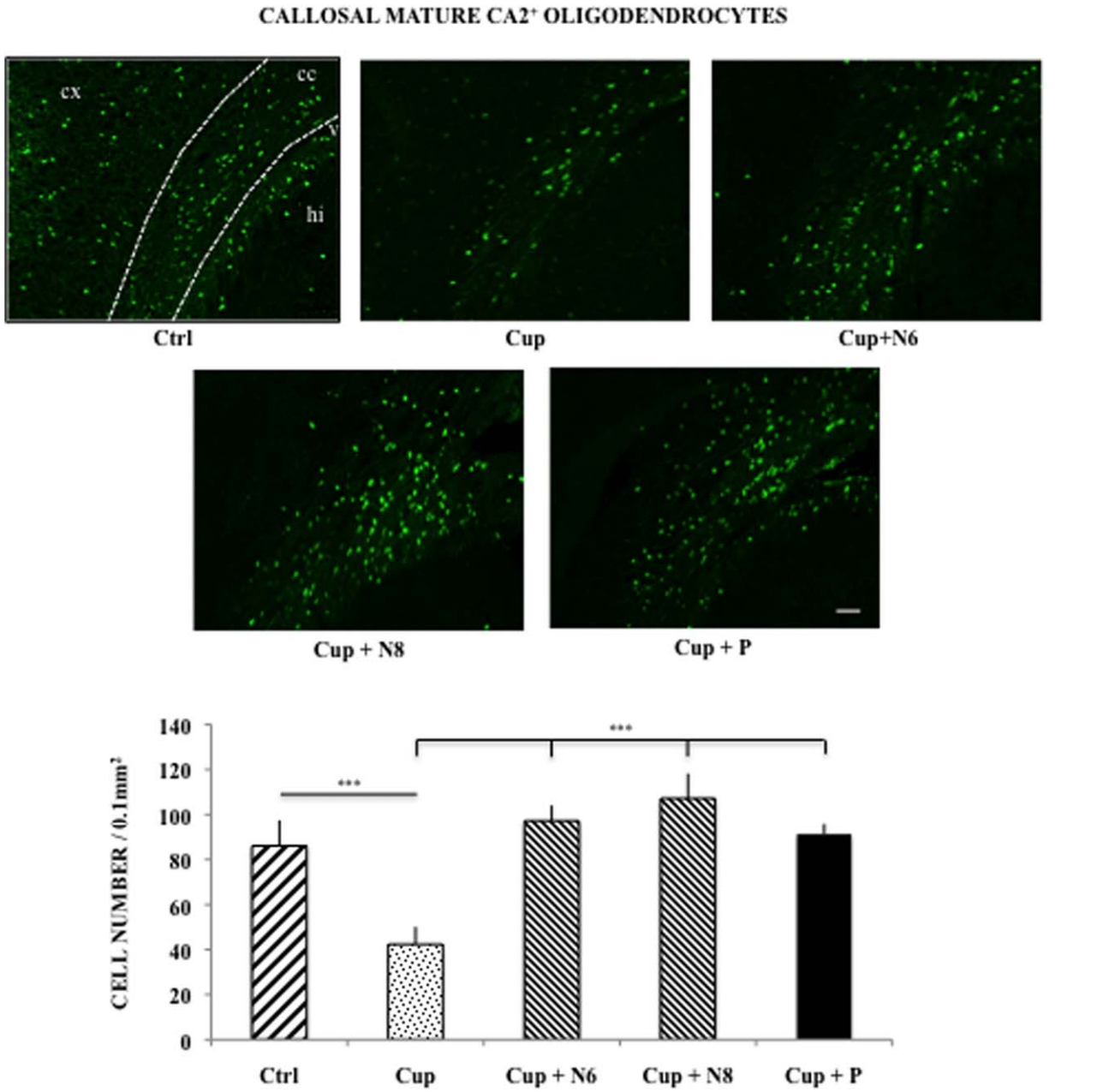

FIGURE 4: Nestorone promoted replenishment of the corpus callosum with mature oligodendrocytes. The administration of cuprizone (Cup) during 12 weeks induced a marked decrease in the number of CA II$^+$ oligodendrocytes within the corpus callosum of intact female mice, when compared to controls, which were not exposed to cuprizone (Ctrl). Treatment during 3 weeks with 6 μg/day (N6) or 8 μg/day (N8) of Nestorone released by osmotic minipumps or Silastic implants of progesterone (P) restored the number of oligodendrocytes ($n = 5–7$ per group). The body of the corpus callosum is highlighted by the white dotted lines. Cx: cortex; CC: corpus callosum; hi: hippocampus; v: lateral ventricule. All data are presented as means ± S.E.M. and were analyzed by one-way ANOVA followed by Bonferroni tests (*$p < 0.001$). Scale bar = 50 μm.**

The number of activated Iba1$^+$ microglial cells was also upregulated during cuprizone-induced demyelination. It decreased to control levels in the cortex after 3 weeks of progesterone or Nestorone, whereas in the corpus callosum, where cuprizone induced a larger increase in microglial cell number, progestogen treatment decreased it by 50% (Figs. 9C and 9D). In summary, both progesterone and Nestorone exert strong anti-inflammatory actions in demyelinated brain structures.

Discussion

Suppressing immune attacks on myelin and reducing neuroinflammation remain the major therapeutic interventions in MS (Kieseier and Stüve, 2011). However, evolution of

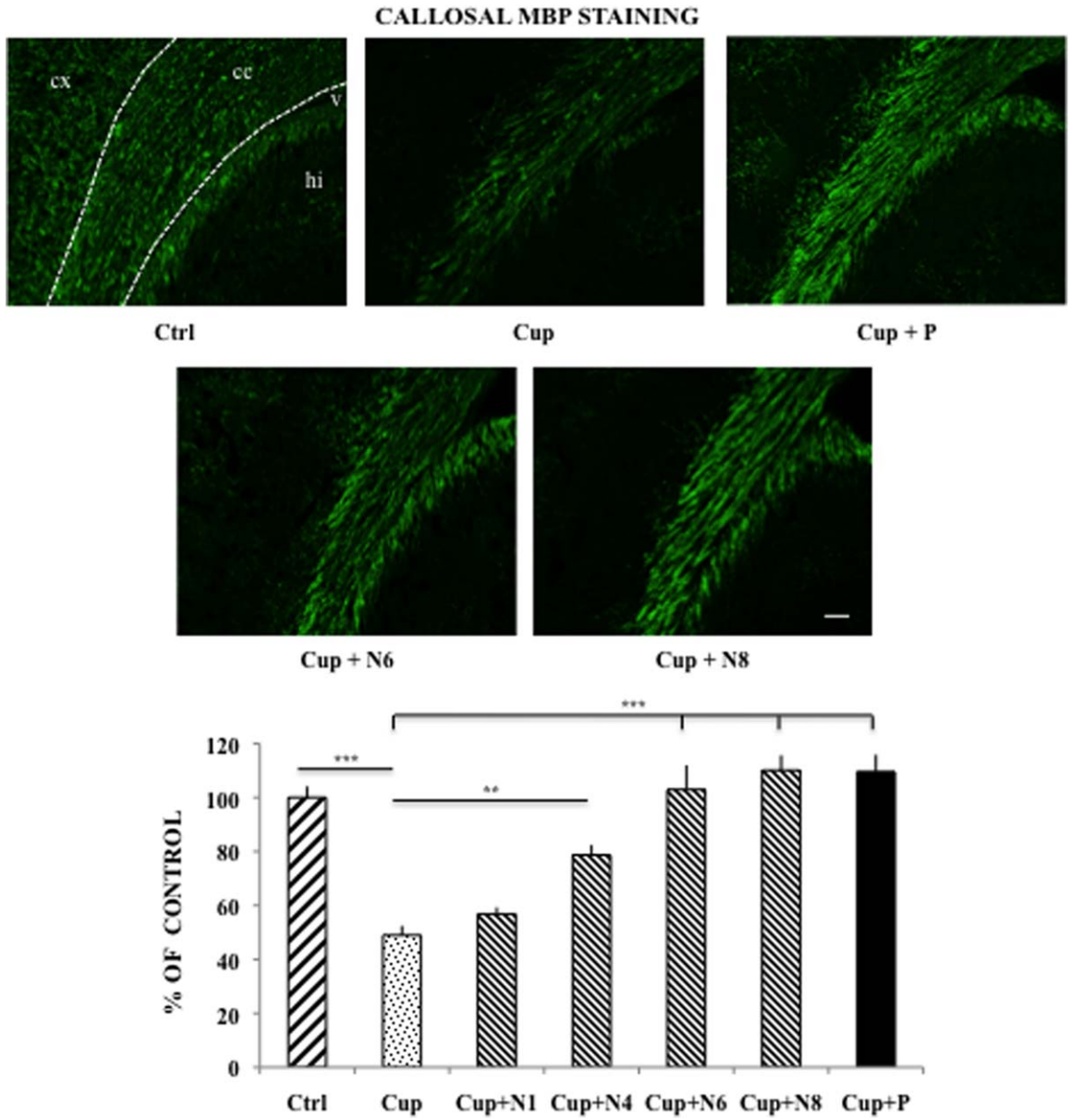

FIGURE 5: Nestorone stimulated the regeneration of MBP$^+$ myelin in corpus callosum. The administration of cuprizone (Cup) during 12 weeks induced a marked decrease in the number of CA II$^+$ oligodendrocytes within the corpus callosum of intact female mice, when compared to controls, which were not exposed to cuprizone (Ctrl). The remyelinating effect of Nestorone was weak when administered for 3 weeks at the dose of 4 μg/day (N4). MBP$^+$ myelin was comparable to Ctrl or progesterone-treated mice (P) for doses of 6 μg/day (N6) or 8 μg/day (N8) of Nestorone ($n = 6$–8 per group). The body of the corpus callosum is highlighted by the white dotted lines. Cx: cortex; CC: corpus callosum; hi: hippocampus; v: lateral ventricule. All data are presented as means ± S.E.M. and were analyzed by one-way ANOVA followed by Bonferroni tests ($P < 0.01$, ***$P < 0.001$). Scale bar = 50 μm.**

relapsing-remitting MS into a chronic-progressive disease course results from the gradual failure of myelin regeneration and the loss of neurons. Therefore, stimulating the endogenous capacity of myelin repair and protecting neurons have become an important therapeutic challenge (Franklin et al., 2012; Martino et al., 2010).

It is now well recognized that steroid hormones have an influence on myelin formation, but their role may be even more important than previously thought. We have recently reported that testosterone and its synthetic analogue 7α-methyl-19-nortestosterone (MENT) promote myelin regeneration in severe demyelinated lesions of the male mouse brain. Moreover, we identified the brain androgen receptor as a target for the remyelinating actions testosterone (Hussain et al., 2013). As in the present study, demyelination and oligodendrocyte depletion of the brain were induced by cuprizone intoxication for 12 weeks, a situation in which no spontaneous regeneration takes place over weeks. This model thus mimics

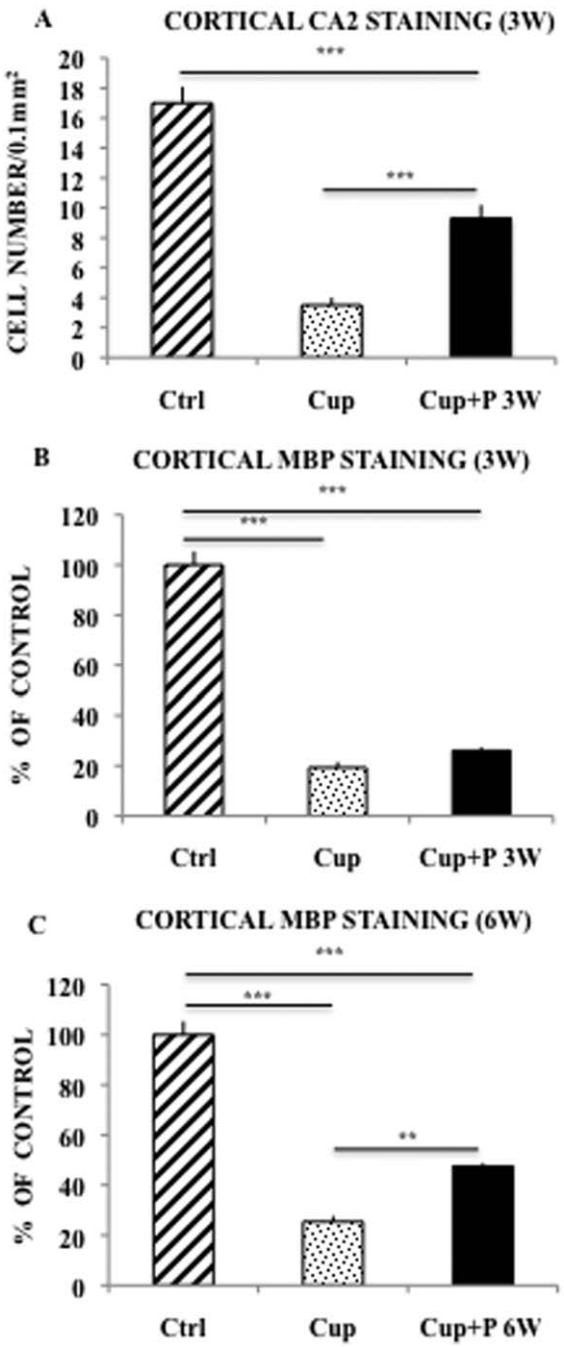

FIGURE 6: Replenishment of oligodendrocytes and remyelination in the cerebral cortex of castrated female mice after 12 weeks of cuprizone intoxication. (**A**) Treatment with progesterone during 3 weeks (P 3W) enhanced the number of CA II$^+$ oligodendrocytes. Results were compared to cuprizone-intoxicated/further untreated mice (Cup) or to control female mice, which were not exposed to cuprizone (Ctrl). (**B**) Cuprizone induced a 80% demyelination, and progesterone treatment during 3 weeks (P 3W) failed to significantly increase MBP immunostaining. (**C**) Treatment with progesterone during 6 weeks (P 6W) increased MBP immunostaining. All data are presented as means ± S.E.M. ($n = 6$–8 per group) and were analyzed by one-way ANOVA followed by Bonferroni tests (**$p < 0.01$, ***$p < 0.001$).

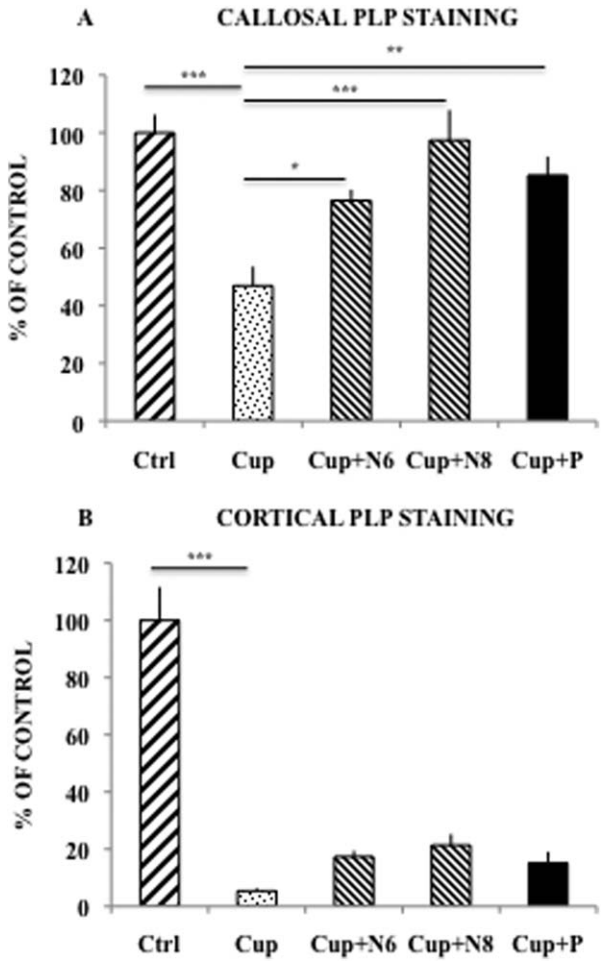

FIGURE 7: PLP immunostaining confirmed data obtained with MBP antibody: indeed Progesterone and Nestorone administered for 3 weeks after the end of a 12 week demyelination favor myelin repair in the corpus callosum (**A**) but not in the cortex, (**B**) as shown by the enhancement of callosal (but not cortical) PLP immunostaining. All data are presented as means ± S.E.M. ($n = 4$–7 per group of intact female mice) and were analyzed by one-way ANOVA followed by Bonferroni tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

the failure of myelin regeneration in chronic MS lesions (Van der Star et al., 2012). It is interesting to emphasize that in this very severe model of persistent demyelination, only the transplantation of progenitor cells or the administration of hormones (testosterone or thyroid hormone) have so far been found to reactivate regenerative processes of myelin (Harsan et al., 2008; Hussain et al., 2013; Mason et al., 2004).

In females, only low levels of testosterone are present, and ovarian steroids may instead play a major role in myelin formation. Progesterone was a likely candidate, as protective effects of the hormone on myelin have been previously demonstrated in EAE and in experimental models of toxin-induced demyelination (Acs et al., 2009; Garay et al., 2007, 2011; Giatti et al., 2012; Ibanez et al., 2004; Yates et al.,

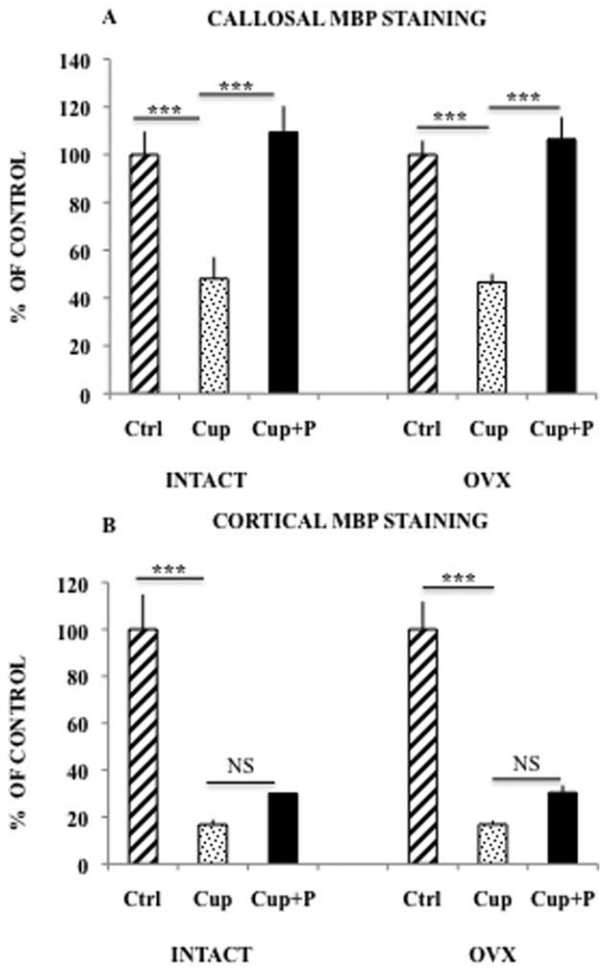

FIGURE 8: Progesterone and Nestorone administered during 3 weeks restored MBP$^+$ myelin in the corpus callosum but not in the cerebral cortex of either intact or ovariectomized female mice. The administration of cuprizone (Cup) during 12 weeks caused a 50% and 80% decrease in MBP immunostaining within the corpus callosum and cerebral cortex, respectively. Cuprizone-induced demyelination was comparable between intact and ovariectomized females. Treatment with progesterone (P) during 3 weeks was sufficient to stimulate the regeneration of MBP$^+$ myelin in the corpus callosum, whatever the presence of ovaries, but failed to significantly increase MBP immunostaining in the cerebral cortex. Results were compared to control female mice, which had not been exposed to cuprizone (Ctrl) ($n = 6$–8 per group). All data are presented as means ± S.E.M. and were analyzed by one-way ANOVA followed by Bonferroni tests (*$p < 0.001$, NS = nonsignificant).**

2010; Yu et al., 2010). Moreover, progesterone promoted the proliferation and differentiation of OPC after spinal cord injury (Labombarda et al., 2009), and both progesterone and Nestorone have been shown to stimulate remyelination in demyelinated organotypic cultures of cerebellar slices (Hussain et al., 2011). Whether progesterone also promotes myelin regeneration *in vivo* after chronic demyelination and the depletion of oligodendrocytes, as does testosterone in males, remained to be explored.

Following a chronic 12 week exposure to cuprizone, Armstrong et al (2006) still observed a progress in demyelination of corpus callosum fibers during a 3 week period after cuprizone intoxication. Instead, another group has reported delayed but efficient remyelination after long-term cuprizone (Lindner et al., 2009). We and others did not observe neither a progress in demyelination nor spontaneous remyelination as late as 6 weeks after cuprizone removal from the diet (Hussain et al., 2013; Mason et al., 2004). Thus depending on the experimental conditions, the severity and persistence of demyelination in the cuprizone model show some variability for still unexplained reasons. In the present study, there were no changes in the number of oligodendrocytes, myelin and inflammation during the 3 weeks following cuprizone withdrawal in the absence of progesterone. However, progesterone treatment during this period efficiently stimulated the recruitment of OPC, the replenishment of mature oligodendrocytes and myelin regeneration in the female mouse brain.

We did not include a group of normal animals treated with progesterone or Nestorone in the absence of cuprizone intoxication, since our previous studies have shown that progesterone is effective only in the injured nervous system. As an example, after a 3 week treatment, progesterone increases the density of mature oligodendrocytes which express Olig 1 transcription factor only in the injured cenral nervous system and not in the control tissue (De Nicola et al., 2009).

We also demonstrated a key role of PR, as 3 weeks progesterone therapy failed to restore oligodendrocytes and MBP$^+$ myelin in PR$^{-/-}$ mice. Importantly, progesterone treatment stimulated less efficiently the myelin regeneration in heterozygous PR$^{+/-}$ mice, strongly suggesting that the receptors may be rate-limiting. Indeed, the loss of a single allele of the PR gene in heterozygous PR$^{+/-}$ mice has been shown to result in a 50% decrease in PR expression in brain and uterus (Liu et al., 2012; Lydon et al., 1995). PR haploinsufficiency has previously been documented for the myelinating and remyelinating effects of progesterone in organotypic brain slices and for the neuroprotective effects of progesterone after transient cerebral ischemia (Ghoumari et al., 2003; Hussain et al., 2011; Liu et al., 2012). This contrasts with the absence of a phenotype for reproductive functions in PR$^{+/-}$ mice (Mani et al., 1997; for a discussion of PR haploinsufficiency, see Schumacher et al., 2014).

The identification of PR as a drug target for remyelination strategies opens new perspectives for the therapeutic use of some progestogens, which have been developed for hormonal contraception or hormone therapies. This is particularly the case for the 19-nor-progesterone derivatives, which have been designed to selectively target PR. One of them, Nestorone (16-methylene-17α-acetoxy-19-norpregn-4-ene-3, 20-dione), not only shows highly selective binding to PR, but

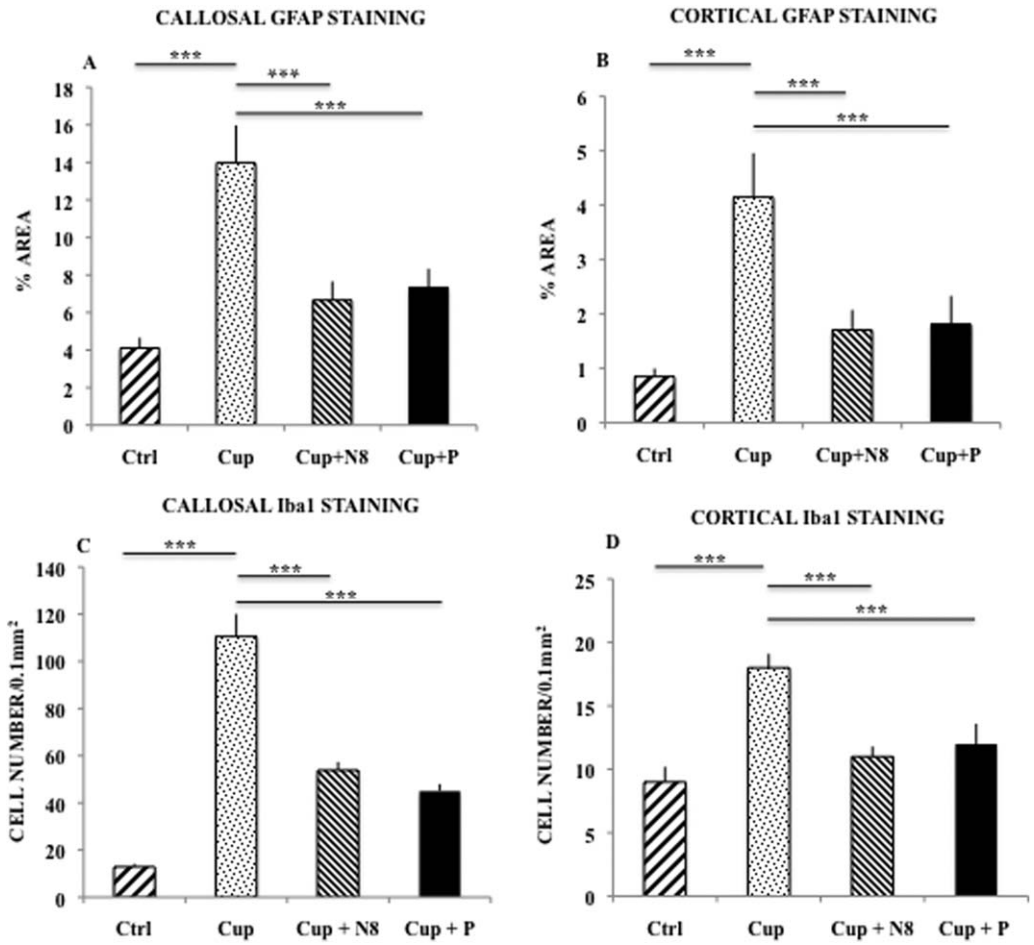

FIGURE 9: Progesterone and Nestorone blunted the increase in reactive GFAP$^+$ astrocytes and (A and B) Iba1$^+$ microglial cells (C and D) in both corpus callosum and cerebral cortex of intact female mice. After 12 weeks of cuprizone (Cup) intoxication, the numbers of GFAP$^+$ astrocytes and Iba1$^+$ microglial cells were markedly increased. Following the removal of cuprizone from the diet, treatment during 3 weeks with 8 μg/day of Nestorone (N8) via mini pumps or Silastic progesterone implants (P), reduced but not abolished) the increase of astrocytes in both structures. It also decreased the number of microglial cells by 50% in the corpus callosum ($n = 6$–8 per group) and nearly to control levels in the cortex. All data are presented as means ± S.E.M. and were analyzed by one-way ANOVA followed by Bonferroni tests (*$p < 0.001$).**

this progestin is also about 100 times more potent than natural progesterone with regard to reproductive functions (Kumar et al., 2000). Two doses of Nestorone (6 or 8 μg/day), which were nine times lower than those of progesterone, stimulated efficiently the replenishment of oligodendrocytes and remyelination in corpus callosum. These results were obtained in female mice with intact gonads, in accordance with the therapeutic condition. A recent study has shown that progesterone protects the brain from demyelination by acting in concert with estradiol (Acs et al., 2009). Ovarian estradiol may thus be necessary for the remyelinating actions of progesterone. However, the formulation of progesterone used in the present study, producing constant diestrus levels of the hormone, promoted oligodendrocyte replacement and myelination with comparable efficacy in either intact or ovariectomized females. This observation shows that the administration of a progestogen alone can be sufficient for stimulating the regeneration of myelin, but it does not rule out additional beneficial or potentiating effects of estrogens. Indeed, beneficial effects of estrogen receptor-mediated actions have been demonstrated in EAE (Polanczyk et al., 2003; Wang et al., 2009; Khalaj et al., 2013; Spence et al., 2011). It is interesting to note that PR can be upregulated by estradiol in many brain regions, although not throughout the CNS (for review: Schumacher et al., 2014).

Progesterone receptors are widely expressed throughout the brain, and they are abundant not only within hypothalamic nuclei involved in the control of reproductive functions, but also in cerebral cortex and subcortical structures (Gofflot et al., 2007; Parsons et al., 1982; Waters et al., 2008). Identification of the neural cell types mediating the remyelinating effects of PR signaling will require the development of

conditional PR-knockout mice. So far, immunohistochemical analyses have shown that PR is mainly expressed in neurons, but also in glial cells (Blaustein and Turcotte, 1989; Jung-Testas et al., 1991; Sakamoto et al., 2001; Warembourg et al., 1989; Waters et al., 2008). In contrast, microglial cells have been reported to be devoid of PR (Sierra et al., 2008).

Whatever the cellular targets of progesterone, an important finding was that progesterone not only promoted the recruitment of oligodendrocytes and myelination in the corpus callosum, but also in the cortical gray matter. New magnetic resonance imaging (MRI) acquisition methods have indeed revealed that in addition to white matter damage, a considerable amount of cortical demyelination occurs during demyelinating diseases such as MS (Geurts et al., 2012). Moreover, demyelinating lesions can already be detected in cerebral cortex at early stages of MS, and they correlate with physical and cognitive impairment (Calabrese et al., 2010, 2012). Under our experimental conditions, the percentage of cortical demyelination was higher than in corpus callosum after 12 weeks of cuprizone, and remyelination was delayed in response to progesterone treatment.

Some progestogens are particularly promising drug candidates for the treatment of demyelinating diseases such as MS because of their multiple beneficial effects: they not only promote the formation of new myelin sheaths, but also exert marked neuroprotective effects. This is particularly important, as neuronal damage and axonal dysfunction are major hallmarks of MS and may play a key role in the progression of the disease toward a chronic form (Friese et al., 2014; Nave and Trapp, 2008). Neurogenic effects of progestogens have been previously described in vivo and in vitro in the hippocampus (Liu et al., 2010). Moreover, neuroprotective effects of progesterone have been demonstrated in rodent models of traumatic brain injury (TBI) and cerebral ischemic stroke (Gibson et al., 2008; Sayeed and Stein, 2009). These experimental studies have provided the basis for clinical trials aimed at investigating the safety and neuroprotective efficacy of progesterone in TBI patients (Stein, 2011; Wright et al., 2007; Xiao et al., 2008). As for the remyelinating effects of progesterone, a key role of PR in its neuroprotective actions has been demonstrated in a recent study. Moreover, a very low dose of Nestorone was shown to promote the resistance of the brain to ischemic damage after transient middle cerebral artery occlusion (Liu et al., 2012).

Myelin regeneration and neuroprotection are complex processes involving a variety of cellular and molecular mechanisms and thus offering multiple therapeutic targets. This may explain why therapies addressing only a single target have fallen behind expectation. Therefore, over the past years has emerged the concept of multifunctional drugs, which promote regeneration and provide protection by addressing multiple targets (Geldenhuys and Van der Schyf, 2013; Youdim et al., 2005). Progesterone and some progestogens are a striking example of multifunctional drugs with promises for the treatment of lesions and diseases of the nervous system. They stimulate the formation of new myelin sheaths, provide protection for neurons, and they also exert anti-inflammatory effects. In our study, treatment with progesterone or Nestorone efficiently modulated astroglial and microglial activation in both corpus callosum and cerebral cortex and this was an important finding. Indeed, neuroinflammation is necessary for oligodendrocyte progenitor differentiation and myelin repair, through the secretion of growth factors by both microglia and astrocytes (Kotter et al., 2005; Moore et al., 2011) and the elimination of myelin debris by microglia. However, strong and prolonged inflammation causes damage to axons and myelin, as seen in MS, in EAE and in models of toxin-induced demyelination.

In MS, inflammatory lesions within the cerebral cortex have been specifically correlated with disability (Calabrese et al., 2012). In EAE, treatment with the tetracycline minocycline caused a concomitant decrease in inflammation, demyelination, and disease activity (Popovic et al., 2002). Moreover, in the cuprizone model, treatment with minocycline reduced numbers of activated microglia in cortical grey matter, diminished demyelination, and prevented disturbances in motor coordination (Skripuletz et al., 2010). However, CNS inflammation appears as a double-edged sword, as it is also part of the stimuli, which promote remyelination (Franklin and Johnson, 1992; Popovich and Longbrake, 2008).

In summary, the present study demonstrates a strong remyelinating effect of progesterone in female mice mediated by its intracellular receptor. While previous work demonstrated a *protective* effect of progesterone, these data support a *therapeutic* effect in an established disease model. We identify PR as a promising therapeutic target for myelin repair, and we show that the potent and PR-selective progestogen Nestorone is a promising therapeutic agent for promoting myelin regeneration. These findings provide the preclinical basis for the therapeutic use of some progestogens already validated for contraception and hormone therapy, in myelin repair strategies, and for clinical trials aimed at assessing their therapeutic efficacy in MS.

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