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ORIGINAL ARTICLE

Progesterone Attenuates Several Hippocampal Abnormalities of the Wobbler Mouse

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It is now recognised that progesterone plays a protective role for diseases of the central nervous system. In the Wobbler mouse, a model of motoneurone degeneration, progesterone treatment prevents spinal cord neuropathology and clinical progression of the disease. However, neuropathological and functional abnormalities have also been discovered in the brain of Wobbler mice and patients with amyotrophic lateral sclerosis. The present study examined the hippocampus of control and afflicted Wobbler mice and the changes in response to progesterone treatment. Mice received either a single progesterone implant (20 mg for 18 days). We found that the hippocampal pathology of the untreated Wobblers involved a decreased expression of brain-derived neurotrophic factor (BDNF) mRNA, decreased astrogliosis in the stratum lucidum, stratum radiatum and stratum lacunosum-moleculare, decreased doublecortin (DCX)-positive neuroblasts in the subgranular zone of the dentate gyrus and a decreased density of GABA immunoreactive hippocampal interneurones and granule cells of the dentate gyrus. Although progesterone did not change the normal parameters of control mice, it attenuated several hippocampal abnormalities in Wobblers. Thus, progesterone increased hippocampal BDNF mRNA expression, decreased glial fibrillary acidic protein-positive astrocytes and increased the number of GABAergic interneurones and granule cells. The number of DCX expressing neuroblasts and immature neurones remained impaired in both progesterone-treated and untreated Wobblers. In conclusion, progesterone treatment exerted beneficial effects on some aspects of hippocampal neuropathology, suggesting its neuroprotective role in the brain, in agreement with previous data obtained in the spinal cord of Wobbler mice.

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that targets the spinal cord and brain stem. However, abnormalities of the limbic system and cerebral cortex are not uncommon in ALS patients. In this regard, frontotemporal dementia and ALS may share an overlapping neuropathology and clinical symptoms (1). Recent findings in TDP-43 proteinopathy have demonstrated that the pathological localisation and/or expression of this protein also involves the brain stem, neocortical and limbic areas of ALS patients (2).

In addition to humans with ALS, brain abnormalities have also been reported in animal models of motoneurone degeneration. Decreased oxidative phosphorylation has been shown in brain mitochondria from mutant Wobbler mice studied at the early and late stages of the disease (3). A decreased function of respiratory chain complex IV in the Wobbler brain was also reported (4). In accordance with findings in presymptomatic and symptomatic ALS patients, Wobbler mice present hyperexcitability of the cerebral cortex as a result of a deficit in the inhibitory GABAergic system and in the vesicular GABA transporter of cortical pyramidal cells (5). GABA efflux is also impaired in Wobbler mice (6). In another ALS model, the superoxide dismutase-1 (SOD1) transgenic mouse, some stages of the disease present a supraspinal pathology, including the thalamus, motor trigeminal nucleus and cerebral motor cortex (7).

The brain abnormalities of ALS patients include deficits in learning and memory, suggesting a dysfunctional hippocampus. Morphologically, there are cytoplasmic inclusions in the granule cells of the

dentate gyrus, neuronal loss in the transtentorial cortex, and degeneration of the outer molecular layer of the dentate gyrus and of the perforant pathway (8). In chronic stages, ALS patients may show episodes of memory loss, imitating Alzheimer's disease (8). The neuroendocrine function of the hippocampus, including the negative-feedback of adrenal steroid hormones, also appears to be affected. Thus, ALS patients show a loss of the cortisol circadian rhythm, as a result of a significant morning increase in circulating cortisol, coupled with a decreased response to stress (9). It was concluded that ALS patients present a dysfunctional central nervous system-hypothalamic-pituitary-adrenal axis (HPA). Abnormalities of the HPA axis and of adrenal steroid secretion are also present in Wobbler mice (10). Wobbler mice show increased basal plasma corticosterone, a hyper-response to stress and a lower binding capacity of hippocampal glucocorticoid receptors (10), which are changes suggesting a centrally-mediated hyperadrenocorticism. In this regard, glucocorticoid hormones make the hippocampus more vulnerable to diseases and stress, or a combination of the two (11,12).

Progesterone plays a neuroprotective role in experimental or human cases of traumatic brain injury, stroke, ischaemia, spinal cord injury, neuroinflammation, neurophatic pain and peripheral neuropathy of traumatic or diabetic origin (13–19). Progesterone treatment also prevents spinal cord neuropathology and the clinical outcome of Wobbler mice (20–23). Among the mechanisms proposed for progesterone neuroprotection, modulation of the GABAergic system has been considered. Progesterone may act directly on GABA synthesising neurones expressing progesterone receptors (24). Moreover, its metabolite allopregnanolone is a potent enhancer of $GABA_A$ receptor activity (25). GABAergic interneurones are responsible for controlling many hippocampal functions, including dentate gyrus neurogenesis (26). Thus, normalisation of the faulty GABAergic system of the Wobbler mouse (5,6) could reinstate missing hippocampal functions.

In the present study, we examined whether progesterone also exerts beneficial effects on the Wobbler mouse hippocampus, and whether progesterone neuroprotection involves common mechanisms in the hippocampus and spinal cord.

We focused on the expression of hippocampal brain-derived neurotrophic factor (BDNF), astrogliosis, the density of doublecortin-positive (DCX+) cells in the dentate gyrus, and GABA expression by interneurones. The results obtained show that, with the exception of the deficit in DCX+ cells, abnormalities of the Wobbler mouse hippocampus are attenuated by progesterone treatment.

Materials and methods

Experimental animals

Male and female breeder mice (NFR/wr) were obtained from the Instituto de Biología y Medicina Experimental mouse colony. Mice were kept under a 12 : 12 h light/dark cycle (lights on 07.00 h), with controlled humidity and temperature (22 °C), and fed standard mice chow with vitamin supplementation (Ensure, Abbott, Zwolle, Holland). The Wobbler phenotype was identified as described previously (27), employing an *Alu*I restriction polymorphism of a Cct4 amplification product for testing the allelic status at the wr locus. Cct4 diagnostic primers and restriction enzymes for genotyping were purchased from Promega (Madison, WI, USA). Genotyping demonstrated that the wr/wr phenotype represented approximately 20% of each litter. Animals were housed in group cages containing two or three Wobblers and one control mouse. This social interaction prolonged the life span and improved the health status of the Wobbler mice (M.-P. Junier, personal communication). In the present experiments, we employed 5–8-month-old Wobbler mice presenting tremor, ambulatory difficulty and slight flexion of proximal limbs, pronounced curved digits, positive clasp knife reflex response, diminished muscle strength and weight loss. Animal of both sexes were used in comparable numbers in all groups because neither the onset, nor the progression of the disease correlated with sex (28). Following light isofluorane anesthesia (Baxter, Guayama, Puerto Rico) a group of Wobbler (wr + /wr +) and control (NFR/NFR) mice were implanted s.c. below the skin of the neck with a single implant of 20 mg of progesterone (Sigma, St Louis, MO, USA) pellet. Compressed pellets of progesterone crystals were made using a manual pellet press and used immediately without storage. The pellet was left in place for 18 days. Eighteen days following progesterone pellet implantation, serum progestins of Wobbler mice showed a 16–20 fold increase (approximately 64 ng/ml) (29), whereas, in the cervical spinal cord, levels were ten-fold higher compared to untreated Wobblers or control mice (R. Guennoun, personal communication). The selected exposure time (18 days) allowed the study of the effects of progesterone on motoneurones and glial cells, although it is not sufficiently long to determine any behavioural or clinical benefits, which required at least 2 months of continuous steroid exposure (22). Animal procedures followed the Guide for the Care and Use of Laboratory Animals (NIH Guide, Instituto de Biología y Medicina Experimental Assurance Certificate # A5072-01) and were approved by the Institute's Animal Care and Use Committee. Efforts were taken to keep the number of animals at a minimum to obtain statistical significance.

In situ hybridisation (ISH) for BDNF mRNA in the hippocampus

ISH for BDNF mRNA was performed as described previously (22,23). Following deep anaesthesia with a mixture of xylazine (6 mg/kg) and ketamine (75 mg/kg), animals were perfused transcardially with 0.9% NaCl prepared in diethylpirocarbonate-treated water, followed by 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer pH 7.2. Brains were removed, post-fixed for 2 h at 4 °C in the same fixative, cryoprotected by immersion in 20% sucrose overnight and kept frozen at −80 °C until used. Cryostat sections (16 μm) of the dorsal hippocampus (Bregma: −1.82––2.18 mm) (29) were fixed in 2% paraformaldehyde, washed with saline-sodium citrate (SCC) buffer (23), dried and acetylated with acetic anhydride. A (^{35}S) dATP-labelled BDNF oligonucleotide probe (Oligos Etc, Inc., Wilsonville, OR, USA) complementary to bp 650–699 of the coding region of mouse BDNF Exon VIII (30,31) was hybridised to the sections in 100 μl of hybridisation cocktail containing: 0.02% Ficoll 400, 0.02% polyvinylpirrolidone, 0.02% bovine serum albumin, 50% formamide, 3 SCC buffer, 10 mM dithiothreitol, 0.1 mg/ml salmon sperm DNA, 1 mM ethylenediaminetetraacetic acid, 4 μg/ml heparin, 0.4 mg/ml tRNA and 10% dextran sulphate. After overnight hybridisation at 42 °C, sections were washed several times in SCC, dried and apposed against Biomax ^{35}S-sensitive films (Kodak, Rochester, NY, USA) for 24 h. Film autoradiograms were anaysed by computed-assisted densitometry employing NIH IMAGE Software (NIMH, Bethesda, USA). Data were expressed as the percentage background obtained from sections exponed to ^{35}S-labelled probe in the presence of a 20 excess nonradioactive oligonucleotide. Densitometric readings were taken from the CA3 pyramidal cell layer and the dentate gyrus. The results are expressed as percentage optical density (mean ± SEM). The BDNF mRNA signal was quantified in six sections per animal (five animals per group).

DCX immunostaining of neural progenitors

Anaesthetised mice were transcardially perfused with 0.9% saline followed by 4% PFA in phosphate-buffered saline (PBS), pH 7.4. After an overnight incubation in 4% PFA, brains were transferred to Tris-buffered saline (TBS), pH 7.4, sectioned frontally at 50 μm using a vibrating microtome and processed for free-floating immunocytochemistry. A DCX antibody was used to label proliferating neuroblasts and immature neurones (32).

Accordingly, coronal brain sections were first exposed to methanol : H_2O_2 (100 : 1) for 10 min at room temperature washed and blocked for 30 min in PBS containing 10% rabbit serum at 37 °C. Sections were incubated overnight with a goat polyclonal anti-DCX antibody (dilution 1 : 250; sc-8066, Santa Cruz Biotechnology, Inc.) followed by a biotinylated anti-goat immunoglobulin (Ig)G made in rabbit (dilution 1 : 200; Sigma) and processed with an ABC kit (Vector Laboratories, Burlingame, CA, USA). For development, we used diaminobenzidine chloride 0.25 mg/ml (Sigma) and 0.05% H_2O_2 at room temperature (25 °C). Sections were finally dried, dehydrated and mounted with Permount (Fischer Scientific, Pittsburgh, PA, USA). Nonspecific staining was assessed in the absence of primary antibody. Six sections per animal corresponding to five animals per experimental group were studied. All positive cells were counted with a 40 objective under an BH-2 microscope (Olympus, Tokyo, Japan). Cell counts were restricted to the subgranular cell layer of the dentate gyrus; quantification was performed using the image analysis software Bioscan Optimas II (Bioscan Optimas, Bioscan Inc., Edmonds, WA, USA).

Glial fibrillary acidic protein (GFAP) staining of astrocytes in hippocampus

The effect of the Wobbler mutation and of progesterone treatment on hippocampal astrocytes was studied by immunofluorescence staining for GFAP. Cryostat sections (16 μm) from the dorsal hippocampus were post-fixed in 4% PFA and kept frozen at −80 °C until used. Sections were defrosted, washed in PBS and washed again in PBS containing 0.5% Triton X100. Slices were preincubated with 3% goat serum in 0.5% PBS-Triton at 37 °C for 10 min and then incubated with a 1 : 250 dilution of the primary GFAP antibody (rabbit polyclonal anti-GFAP, catalogue number G-9269; Sigma) made in Triton-PBS buffer and 2% goat serum to block nonspecific binding. After incubation for 18–20 h at 4 °C, a goat anti-rabbit IgG conjugated to Alexa Green 488 (dilution 1 : 1000) (Invitrogen, Molecular Probes, Eugene, OR, USA) was added in Triton-PBS and 2% goat serum solution. After 1 h of incubation in the dark, sections were washed, dried, mounted with Fluoromount-G (0100-01; Southern Biotech, Birmingham, AL, USA) and examined under a confocal Nikon Eclipse E 800 microsope equipped with Nikon 11691 photographic equipment (Nikon, Tokyo, Japan).

Images taken with the confocal microscope were analysed using IMAGE J (NIH, Bethesda, MD, USA) at × 600. The number of GFAP+ astrocytes per unit area was quantified in the hilus, the stratum lucidum, stratum radiatum and stratum lacunosum moleculare corresponding to the CA1, CA2 and CA3 pyramidal cell layers. Astrocytes were counted in five sections comprising the right and left hemispheres from five animals per experimental group.

GABA staining of interneurones

Immunostaining for GABA in interneurones was carried out on 60-μm coronal sections of dorsal hippocampus obtained with a vibratome. Free-floating sections were treated with 0.3% H_2O_2 in methanol/PBS (50 : 50) to inhibit endogenous peroxidase, washed and blocked with 10% horse serum. Sections were incubated with a 1 : 750 dilution of a GABA antibody (anti-GABA 9 made in rabbit; kind gift of Dr Peter Somogyi, Anatomical Neuropharmacology Unit, Oxford, UK). Thereafter, sections were incubated with a 1 : 1000 dilution of an anti-rabbit antibody conjugated to Alexa Fluor 488. After several washes, sections were mounted with Fluoromont G and examined with the confocal microscope.

Double immunofluorescence staining for NeuN and GABA

The phenotype of interneurones and neurones of the dentate gyrus was investigated using double immunostaining for NeuN and GABA. Free-floating sections were incubated with the monoclonal NeuN antibody (anti-Neuronal Nuclei MAB 2377, Chemicon-Millipore, Billerica, CA, USA) at a 1 : 1000 dilution and with the polyclonal rabbit anti-GABA antibody (dilution 1 : 750). Incubations with the primary antibodies lasted for 2 days and slices were rinsed three times in TBS 0.1% Triton X-100 for 15 min before application of the second antibodies: goat anti-mouse IgG conjugated to Alexa Fluor 555 (dilution 1 : 1000, Invitrogen, Molecular Probes) and goat anti-rabbit IgG conjugated to Alexa Fluor 488 (dilution 1 : 1000; Invitrogen, Molecular Probes). Incubation with second antibodies was followed by three rinses in TBS. Sections were mounted with Fluoromont G and kept in the dark at 4 ° C until analysis by confocal microscopy. Double-labelled cells were examined under a Nikon Eclipse E 800 confocal scanning laser microscope. Images were acquired sequentially in a line-scanning mode through an optical section of 0.5 μm in the z-axis, and merged using Nikon EZC1 2.1 software. Quantification was performed using the Bioscan Optimas II system. Results were expressed as% of GABA immunofluorescent cells showing NeuN immunofluorescense staining.

Statistical analysis

All results are expressed as the mean ± SEM. Data were analysed by one-way ANOVA followed by a post-hoc Newman–Keuls test. Statistical analysis was performed with PRISM 4 (GraphPad software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Progesterone effects on hippocampal BDNF mRNA were studied in 5–8-month-old symptomatic Wobbler mice and age-matched controls. This disease stage was selected because spinal cord BDNF mRNA expression is more sensitive to progesterone than at other ages (23).

Film autoradiography following ISH (Fig. 1A) clearly showed decreased BDNF mRNA expression in untreated Wobblers (WR), which was recovered by progesterone treatment (WR + PROG). Quantitative data obtained from the composite optical density of all film autoradiograms from all animals demonstrated, in two analysed hippocampus regions, a reduced expression of BDNF mRNA in the dentate gyrus ($P < 0.001$; Fig. 1B) and pyramidal CA3 region ($P < 0.01$; Fig. 1C) of Wobblers (dark columns) compared to control mice (longitudinal line columns). Progesterone treatment of the mutant mice (grey columns) significantly rescued BDNF mRNA in both regions ($P < 0.001$ for dentate gyrus, Fig. 1B; $P < 0.01$ for CA3 region, Fig. 1C). In control mice, progesterone induced a non-significant increase in the CA3 region (Fig. 1B) and did not change BDNF mRNA in the dentate gyrus (Fig. 1C).

Previous results have shown that spinal cord astrogliosis was a typical finding of the Wobbler disease, and that progesterone treatment markedly reduced astrocyte reactivity (23). To determine whether similar effects occurred in the hippocampus, the number

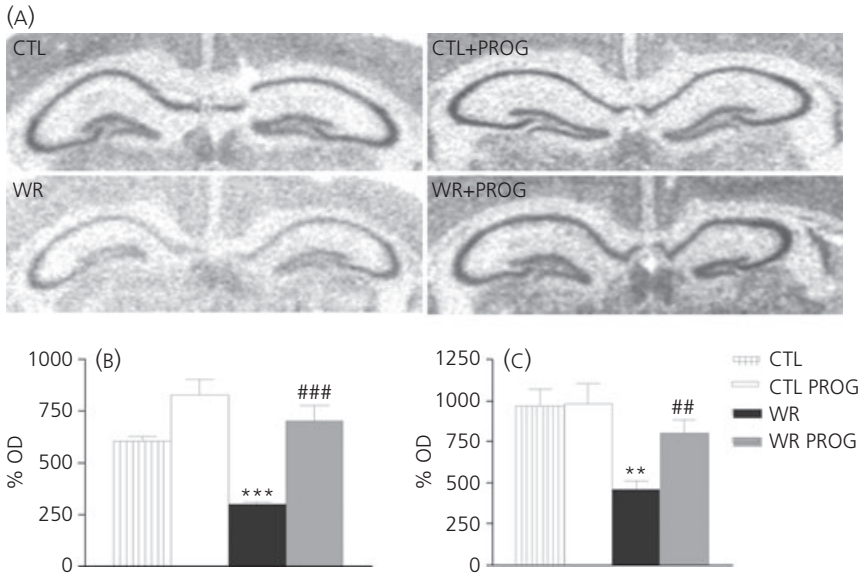

Fig. 1. (A) Representative film autoradiograms showing oligonucleotide probe hybridised to brain-derived neurotrophic factor (BDNF) mRNA in hippocampus. Images show low BDNF signalling in a Wobbler (WR) compared to a control mouse (CTL). Progesterone showed no effect in a control mouse (CTL + PROG), although it greatly increased the BDNF mRNA signal in a Wobbler (WR + PROG). (B, C) Optical density (OD) measurements of film autoradiograms representing BDNF mRNA expression in the hippocampus. The graphs show the decreased BDNF expression in the Wobbler (dark columns) dentate gyrus (B, $^{***}P < 0.001$) and CA3 pyramidal region (C, $^{**}P < 0.01$) compared to NFR/NFR control mice (longitudinal columns). Progesterone treatment of Wobbler mice significantly increased the OD for BDNF mRNA in both regions versus untreated Wobblers (B, $^{###}P < 0.001$ for dentate gyrus; C, $^{##}P < 0.01$ for CA3 region). Percentage OD measurements were analysed on films obtained from the combined right and left dorsal hippocampus from six sections per animal (five animals per group).

of GFAP+ astrocytes was counted in the hilus and the stratum lucidum, stratum radiatum and stratum lacunosum moleculare of control mice, controls receiving progesterone, Wobbler mice and Wobblers receiving progesterone. GFAP+ astrocyte density, computed from the numbers of all sections from all animals, was highly increased in Wobbler mice for the three stratums corresponding to CA1, CA2 and CA3 subregions (Fig. 2A–C, dark columns). Progesterone treatment significantly down-regulated Wobbler astrogliosis in the three stratums corresponding to the CA1, CA2 and CA3 regions (Fig. 2A–C, grey columns). By contrast, hilar astrocyte density was similar in untreated Wobblers or those receiving progesterone (data not shown). The images of Fig. 2(D) represent, from top to bottom, GFAP+ astrocytes in a control mouse hippocampus (CTL), the pronounced astrogliosis of the Wobbler (WR) and its reduction in a Wobbler receiving progesterone (WR PROG).

GABAergic interneurones and granule cells are important for hippocampal function. Thus, the neuronal phenotype of steroid-naïve control and Wobbler mice and those receiving progesterone were compared using double immunofluorescence staining for NeuN and GABA markers. Double-labelled cells (NeuN+/GABA+) comprised approximately 30% of the total NeuN+ cells, indicating that one-third of interneurones expressed the gabaergic phenotype. Because Wobbler mice present a diminished GABAergic tone (5,6), we investigated whether GABA immunostaining was changed by the progesterone treatment of Wobbler mice. In a control mouse (CTL; Fig. 3A) several GABA+ interneurones were observed in the stratum lacunosum moleculare. By contrast, fewer GABA+ profiles were observed in a steroid-untreated Wobbler mice (WR). There was a moderate rebound of GABA+ cell density in the stratum lacunosum moleculare of the Wobbler receiving progesterone (WR + PROG). Quantitative analysis (Fig. 3B) of the composite numbers of all sections from all animals demonstrated that, in control mice, GABA+ cells amounted to 116.4 ± 10.1/unit area (mm^2), which are significantly higher values than for Wobbler mice (70.7 ± 5, $P < 0.01$). Progesterone treatment of the Wobblers significantly increased GABA+ interneurones to 102.2 ± 2.2/unit area ($P < 0.001$ versus untreated Wobblers) (Fig. 3B).

Because hippocampal GABA directly influences neuronal progenitors of the subgranular zone of the dentate gyrus (26), a final step of our investigation determined the density of DCX-immunoreactive cells in the four experimental groups. In control mice, several DCX+ cells populated the subgranular zone, with staining of bodies and cell processes (Fig. 4A, CTL). The composite number of DCX+ cells in the dentate gyrus from all sections from all control animals was 5208 ± 1036 (Fig.4B, longitudinal line column). This number was slightly but not significantly reduced by progesterone treatment of controls (4443 ± 1351), although staining of cell processes was weaker to absent in the controls receiving progesterone (Fig. 4A, CTL + PROG). A pronounced reduction of DCX+ cells was obtained in steroid-naïve Wobblers (3036 ± 269) ($P < 0.01$ versus CTL; Fig. 4B; dark columns), which remained unchanged following progesterone treatment (2250 ± 163, grey column). The photomicrographs of Fig. 4 (A) show, at the morphological level, scant DCX immunoreactive cells in the subgranular zone of both untreated Wobblers (WR) and Wobblers receiving progesterone (WR + PROG).

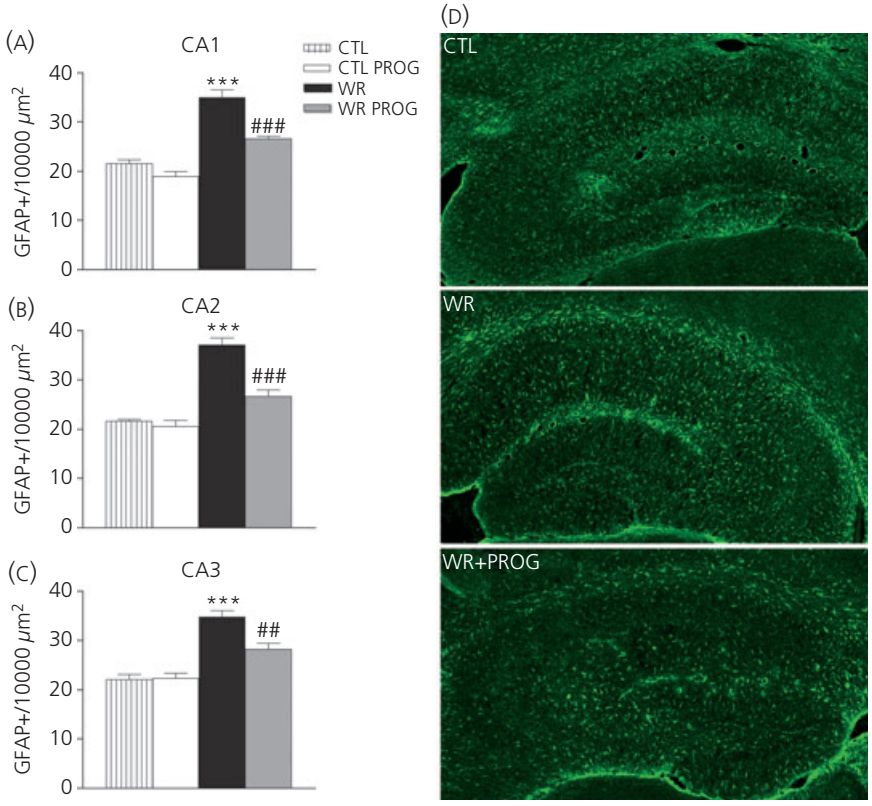

Fig. 2. Computerised image analysis of the number of glial fibrillary acidic protein-positive (GFAP+) cells per unit area in combined strata (lucidum, radiatum and lacunosum moleculare) corresponding to the CA1, CA2 and CA3 hippocampal regions from control (longitudinal line columns), control + progesterone (white columns), Wobbler (dark columns) and Wobbler + progesterone treated mice (grey columns). (A) In CA1, (B) CA2 and (C) CA3 regions, GFAP+ astrogliosis was observed in Wobblers (***$P < 0.001$) compared to control and control + progesterone-treated mice. Progesterone treatment reduced astrogliosis in the CA1 and CA2 regions ($^{###}P < 0.001$) and the CA3 region ($^{##}P < 0.01$) compared to untreated Wobblers. (D) Representative GFAP immunfluorescence staining of the dorsal hippocampus of a control (CTL), Wobbler (WR), and a progesterone-treated Wobbler mouse (WR + PROG). Intense GFAP+ astrogliosis was shown in strata below the CA1, CA2 and CA3 pyramidal cell regions of Wobbler compared to the control mouse. Reactive astrocytosis of the Wobbler was down-regulated by progesterone in all regions. Magnification × 400.

Discussion

Progesterone neuroprotection in the Wobbler mouse spinal cord has been reported previously (20–23,33). In the present study, we investigated whether the effects of progesterone also take place in the hippocampus of this animal model. Mice in the symptomatic period of the disease were chosen because previous studies have demonstrated that this stage is more responsive to progesterone (23,33). First, our results show that the hippocampus was not spared from neurodegeneration. Hippocampal pathology of the Wobbler included: (i) decreased expression of BDNF mRNA; (ii) astrogliosis in the stratum lucidum, stratum radiatum and stratum lacunosum-moleculare; (iii) decreased number of DCX+ neuroblasts in the subgranular zone of the dentate gyrus; and (iv) decreased density of GABA immunoreactive interneurones. The present study also established that progesterone treatment attenuated several of these hippocampal abnormalities. Thus, progesterone increased BDNF mRNA expression in the CA3 pyramidal cell layer and gyrus dentatus; decreased GFAP+ astrogliosis in several hippocampal regions; and increased GABA immunorestaining of interneurones.

Therefore, in agreement with previous findings in the spinal cord (22,23), progesterone markedly stimulated BDNF mRNA in the hippocampus. This effect may be crucial for some BDNF-regulated functions of hippocampus, including synaptic transmission, neuronal survival, dendritic spine plasticity and long-term potentiation (34–37). The diminished BDNF mRNA in the hippocampus of untreated Wobblers may be the consequence of the ongoing neurodegeneration. However, inhibition of BDNF mRNA by glucocorticoids (38) should also be considered, in view of the hyperadrenocorticism reported in Wobbler mice (10). Progesterone up-regulation of BDNF mRNA in Wobbler mice may be linked to protective effects of progesterone on this structure. Several reports have shown that progesterone positively modulates BDNF in different experimental models, including spinal cord neurodegeneration, traumatic injury of the spinal cord, cultured cerebral cortical explants and c6 rat glioma cells (28,39–41). A protective role of exogenous BDNF in the Wobbler has already been shown *in vivo*; in this case, BDNF increases survival and muscle strength (42–44). Thus, up-regulation of BDNF may be part of the mechanisms involved in progesterone neuroprotection.

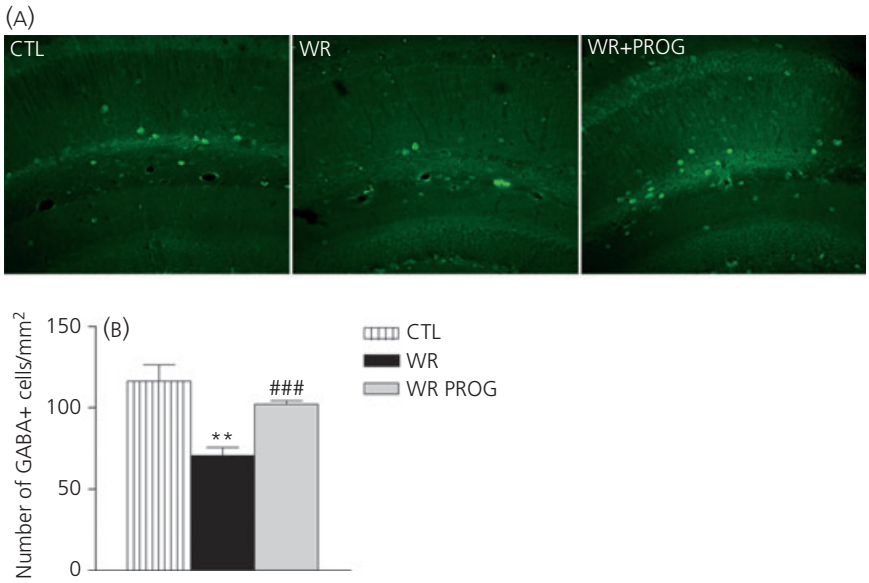

Fig. 3. (A) Immunofluorescence staining for GABA in a representative control mouse (CTL), Wobbler (WR) and a progesterone-treated Wobbler mouse (WR + PROG) The photomicrographs show fewer glial fibrillary acidic protein-positive (GFAP+) interneurones in the stratum lacumosum moleculare of Wobbler hippocampus compared to a control mouse, whereas progesterone treatment of the Wobbler increases GABAergic cell density. Magnification × 200. (B) Quantitative analysis of the number of GABA immunoreactive cells in the stratum lacunosum moleculare of control and Wobblers without or with progesterone treatment. GABA+ cells of control mice (longitudinal line column) were significantly higher than in Wobbler mice (dark column, $^{**}P < 0.01$ versus control). Progesterone treatment of the Wobblers significantly increased GABA + interneurones (grey column, $^{###}P < 0.001$ versus untreated Wobblers).

In parallel with findings obtained in the spinal cord (20,27,33,45), the hippocampus of Wobbler mice developed an intense astrocytic reaction with a strong expression of GFAP. Astrogliosis may be a secondary response to neuronal degeneration, although it has also been considered a cell-autonomous event of the Wobbler mouse (45,46) and of the SOD1 transgenic mouse model (47). Wobbler astrocytes show abnormal differentiation and morphology, differential responses to several mitogens in tissue culture and a reduced expression of glutamine synthetase (46,48,49), suggesting that the high density of astrocytic processes in the Wobbler mouse may cooperate or even trigger neuronal death. In ALS patients, astrogliosis is considered toxic for motoneurones because reactive astrocytes release proinflammatory mediators and prostaglandins and secrete proapoptotic factors (47,50). We found that the progesterone reduction of GFAP+ astrocytes extended to all hippocampal regions of Wobbler mice. Thus, one way to hold back the progression of the Wobbler disease would be to block hippocampal astrogliosis. Progesterone proved to be highly effective in this respect.

An additional alteration of the Wobbler hippocampus involved GABA immunoreactive neurones, which may represent a vulnerable neuronal population. As already pointed out, a decreased GABAergic system has been observed in the Wobbler brain (5,6). GABA released from interneurones directly regulates several steps of adult neurogenesis, including the proliferation of neural progenitors, migration and differentiation of neuroblasts, and synaptic integration of newborn neurones (26,51). Therefore, we speculated that a reduced GABAergic transmission could be related to impaired hippocampal neurogenesis of the Wobbler. However, although progesterone showed a positive impact on the density of GABA immunoreactive interneurones, it did not increase the number of DCX+ neuroblasts and immature neurones.

The failure to recover DCX-immunopositive neural progenitors of the Wobbler's dentate gyrus should be understood on the premise that the hormonal regulation of hippocampal neurogenesis is a multifaceted issue. In cultured neural progenitors from the rat hippocampus, progesterone stimulates neurogenesis (52). The presence of progesterone receptor mRNA in the dentate gyrus also suggests that progesterone directly influences hippocampal neurogenesis (53). However, when given with oestradiol, progesterone inhibits the effect of the former on cell proliferation (54). Although the effects of progesterone can be direct, it is readily metabolised to allopregnanolone, a purportedly neuroprotective factor (16,19). However, allopregnanolone has been reported to either decrease (55) or increase (19) neurogenesis in adult rodents. Certain pathological conditions bring further complexity to this matter. For example, in traumatic brain injury, where the number of surviving immature neurones increases, progesterone reduces this effect (56). Similarly, progesterone-treatment after stroke suppresses ischaemia-stimulated proliferation of progenitor cells (57). Additional factors interfering with neurogenesis are the glucocorticoids, which circulate in high amounts both in Wobblers and ALS patients (9,10). Glucocorticoids show strong anti-proliferative and anti-neurogenic effects (53,58). Therefore, additional studies are required to elucidate why DCX+ neuroblasts are depressed in Wobbler mice, as well as the hormonal regulation of this process. In this regard, the neurodegeneration context, excess circulating glucocorticoids and the dose of progesterone administered may play undefined roles in this process.

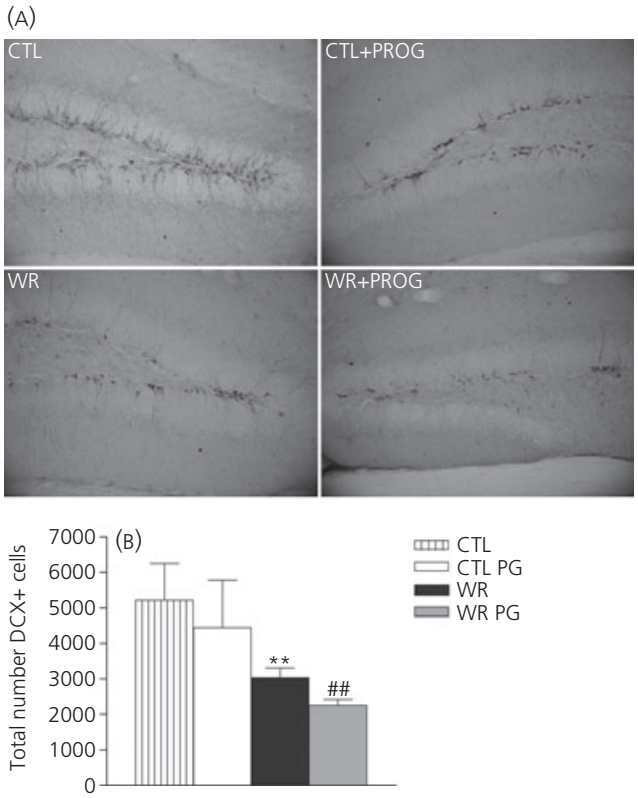

Fig. 4. (A) Doublecortin (DCX) immunoreactive neuronal progenitors in the subgranular zone of the dentate gyrus. Profuse staining of cell bodies and processes appeared in a control mouse (CTL), in contrast to the low staining of cell bodies and processes of the Wobbler (WR). Progesterone treatment diminished the staining of neurites in a control mouse (CTL + PROG), and did not change the low DCX staining of the Wobbler (WR + PROG). Magnification × 400. (B) Quantitative analysis of the number of DCX immunoreactive cell bodies in the subgranular zone of the dentate gyrus. Wobblers without treatment presented reduced density of DCX + profiles (dark columns, $^{**}P < 0.01$ versus control). Progesterone treatment of the Wobblers significantly decreased DCX + cells (grey column, $^{\#\#}P < 0.01$ versus untreated Wobblers). Data were taken from seven sections per animal corresponding to four or five animals per experimental group.

In summary, progesterone treatment of Wobbler mice exerted beneficial effects on some aspects of hippocampal neuropathology, encompassing the stimulation of a neurotrophic factor, holding back astrogliosis, and increasing the expression of GABA-containing interneurones. These effects take place in the brain at the time that several abnormalities of the Wobbler spinal cord are restored. However, the present study has demonstrated the ineffectiveness of progesterone in bringing back the neurogenic deficit of the hippocampus. We suggest that combining progesterone with other steroids showing neurogenic potential and/or glucocorticoid antagonists may help to solve this issue. Thus, animal models of neurodegeneration appear to be well suited for testing novel pharmacological strategies in disorders involving the spinal cord and the limbic brain.

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