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Research report

Progesterone treatment reduces NADPH-diaphorase/nitric oxide synthase in Wobbler mouse motoneuron disease

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Abstract

Previous work demonstrated that progesterone (PROG) treatment attenuates morphological, molecular and functional abnormalities in the spinal cord of the Wobbler (Wr) mouse, a genetic model of motoneuron degeneration. Wr mice show a marked up-regulation of the nitric oxide synthesizing enzyme (NOS). Since nitric oxide is a highly reactive species, it may play a role in neuropathology of Wr mice. We now studied if PROG neuroprotection involved changes of NOS activity in motoneurons and astrocytes, determined by the nicotinamide adenine dinucleotide phosphate-diaphorase (NADPHD) histochemical reaction. Two and four-month-old Wr mice at the progressive and stabilization stages of the disease, respectively, and their age-matched controls were left untreated or received a single 20-mg PROG pellet for 18 days. PROG reduced the high number of NADPHD-active motoneurons and white matter astrocytes in 2-month-old Wr mice but was unable to change the low number of NADPHD-active motoneurons in 4-month-old Wr mice or astrocytes in this age group. A large number of motoneurons in 2-month-old Wr mice showed a vacuolated phenotype, which was significantly reverted by PROG treatment. In summary, PROG treatment during the early symptomatic stage of the disease caused a significant reduction of NADPHD-active motoneurons and astrocytes and also reduced vacuolated degenerating cells, suggesting that blockade of NO synthesis and oxidative damage may contribute to steroid neuroprotection.

Theme: Endocrine and autonomic regulation
Topic: Neuroendocrine regulation: other

Keywords: Progesterone; Neuroprotection; Wobbler mouse; Nitric oxide synthase; Motoneuron disease

1. Introduction

Traditionally, the effects of progesterone (PROG) in the brain have been associated with control of sex behavior and reproductive functions. However, trophic and protective roles for PROG in the peripheral and central nervous system are now widely recognized [27,37]. In peripheral nerves, PROG increases formation of new myelin sheets after lesion, prevents myelin abnormalities of aging rats and stimulates the activity of the promoters of myelin genes [2,9,21]. Centrally, PROG facilitates cognitive recovery and prevents neurodegeneration after cortical contusion, cerebral edema, spinal cord trauma and axotomy [31–33,38,40].

In previous work, we have shown that PROG regulates some key features of neuronal function in rats with spinal cord injury and in the Wobbler (Wr) mouse, a genetic model of spinal cord neurodegeneration [12,13,24]. This genetic mutant presents neuronal loss, astrogliosis and microglia

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activation in the spinal cord and is considered a model for amyotrophic lateral sclerosis (ALS) and infantile spinal muscular atrophy [4,28,43].

In clinical affected Wobbler mice, PROG treatment prevents motoneuron loss and normalizes the mRNA levels of Na, K-ATPase subunits and of the growth-associated protein GAP-43 [13,14]. In conjunction with these data, mice show improvement of life span and muscle strength, suggesting that PROG rescues motoneurons from degeneration and death. The motoneuron degeneration of Wobbler mice is manifested by a dramatic vacuolation of the endoplasmic reticulum and Golgi system and the presence of ruptured mitochondria with damaged cristae [28]. This motoneuron pathology resembles the type II or cytoplasmic form of cell death [5]. Extensive cytoplasmic vacuolation also exists in SOD1 transgenic mice, in which excess formation of free-radicals damages the cell membranes [16,22]. In Wobbler mice, participation of oxidative stress is supported by the increased generation of free radicals by mitochondria and detection of the lipid peroxidation product 4-hydroxynonenal in motoneurons [7,15]. Participation of oxidative stress is also supported by arrest of motoneuron degeneration and retardation of disease progression when Wobbler mice received antioxidant agents, nitric oxide inhibitors or the antioxidant steroid U-74389F [1,10,11,19,20,41].

An interesting feature of Wobbler mice spinal cord is the overproduction of nitric oxide due to the high activity of nitric oxide synthase (NOS) [6]. This finding has also been reported in patients with ALS [29] and in SOD1 transgenic mice [36]. In Wobbler mice, increased number of NOS/nicotinamide adenine dinucleotide phosphate-diaphorase (NADPHD) active motoneurons are found early but not at very late stages of the disease [6]. NO-mediated neurotoxicity becomes manifest after coupling with superoxide anion to generate peroxinitrite and by direct inhibition of several components of the electron transport chain [30,39].

In this work, the end point of PROG treatment was the NADPHD, since in agreement with Clowry and McHanwell [6], we also found strong staining for NADPHD in Wobbler mice spinal cord [11]. Since PROG modulates NADPHD activity in spinal cord after injury [23], we speculated that a similar effect might take place in Wobbler mice. Two periods of the disease were chosen, corresponding to the progressive and stabilizing stages when reportedly, the enzyme activity is high and low [4,6]. Histochemical data were correlated with the percent of motoneuron vacuolation in naïve and PROG-treated Wobbler mice. To better understand steroid mechanisms, the effect of the antioxidant glucocorticoid methylprednisolone (MP) [17,18] on NADPHD was compared to that of PROG. The results suggest that attenuation of NADPHD activity in motoneurons and astrocytes may be part of the mechanisms of PROG-induced neuroprotection in Wobbler mice.

2. Materials and methods

2.1. Experimental animals

Normal phenotype male and female breeder NFR/wr mice were originally obtained from The Animal Center, National Institutes of Health (Bethesda, MD, USA, courtesy of Dr. Carl Hanson), and mated in our Institute. Homozygous Wobbler mice (wr/wr) of two different ages were used. Two-month-old mice—stage 2 of Yung et al. [43], progression stage of Boillée et al. [4]—presented flexion of forelimb digits and slight tremor. Four-month-old mice—stage 3 of Yung et al. [43], stabilizing stage of Boillee et al. [4]—presented reduced body weight, intense tremor, ambulatory difficulty, curled wrists and marked muscle atrophy. Groups of 2- and 4-month-old control or Wobbler mice remained untreated or received a single 20-mg pellet of PROG s.c. under light ether anesthesia. For both groups of mice (2 and 4 months old), dose of PROG, method of administration and length of treatment were similar to those used previously [13]. In the PROG experiment, the following four groups were studied for each age period (2- and 4-month-old mice): (1) control mice; (2) controls + PROG; (3) Wobbler mice; (4) Wobblers + PROG. In another experiment, groups of 2-month-old control or Wobbler mice remained untreated or received i.p. MP 30 mg/kg day for 6 days. In the MP experiment, three groups of 2-month-old mice were analyzed: (1) control mice; (2) Wobbler mice; (3) Wobblers + MP. Two and a half weeks after PROG pellet implantation or at day 7 following continuous MP treatment, animals were deeply anesthetized and perfused transcardially with 0.9% NaCl; the spinal cord was removed following dorsal laminectomy. Cervical spinal cord C_2–C_4 segment and C_4–C_6 segment were used for NADPHD histochemistry and counting of vacuolated cells, respectively. All procedures followed the National Institute of Health Guide for the Use and Care of Laboratory Animals (National Institute of Health Guide, Instituto de Biología y Medicina Experimental, Assurance Certificate No. A5072-01) and were approved by the Institute's Animal Care and Use Committee.

2.2. NADPHD histochemistry and quantitation

A slight modification of the method of Vincent and Kimura [42] was employed to determine NADPHD activity, as previously adapted for the Wobbler mouse spinal cord [11]. Cryostat sections of C_2–C_4 segment of the cervical spinal cord (16 μm) were fixed by immersion in 2% paraformaldehyde in 0.1 M phosphate buffer pH 7.2 during 6 min at 4 °C. After fixation, the sections were rinsed twice in phosphate-buffered saline (PBS) and incubated in a solution of 0.1 M Tris–HCl buffer pH 7.4 containing 0.3% Triton X-100, 0.2 mg/ml of nitroblue tetrazolium (Sigma, St. Louis, MO, USA), 2.7 mg/ml L-malic acid (Sigma) and 1 mg/ml of β-NADPH (Sigma). After keeping

the reaction in the darkness during 90 min at 37 °C, it was stopped by two washes in PBS at room temperature. Sections were then dehydrated briefly in ethanol, dried and coverslipped with Permount. A negative histochemical reaction resulted in the absence of NADPH.

A computer-assisted image analysis system (Bioscan Optimas VI, Edmonds, WA, USA) was used to determine the number of motoneurons or astrocytes per unit area (mm^2) exhibiting blue formazan deposits in perikaryon because of NADPHD histochemical reaction [35]. Spinal cord regions were localized following the stereotaxic atlas of the mouse central nervous system [26]. NADPHD-active motoneurons were counted in Lamina IX on both sides of the ventral horn, whereas astrocytes were counted in white matter of the corticospinal tract (CST) and ventrolateral funiculus (VLF). Sections were observed with an Olympus BHS optic microscope equipped with a Panasonic GP-KR222 video camera. Data corresponding to 12–15 sections per animal ($n=4–7$ animals per group) were combined to give an independent mean, and the animals were used as independent variables [12]. Double counting was unlikely because a minimum of 50-μm spacing was kept between adjacent sections. Photography was carried out in a Zeiss Axioplan optic microscope equipped with an automatic MC 80 camera.

2.3. Quantitative analysis of the number of vacuolated cells

Cervical spinal cords corresponding to $C_4–C_6$ segment from Wobbler mice treated with PROG, without treatment, as well as control animals were fixed in 10% formalin, and embedded in paraffin. Five-micrometer sections were stained with Cresyl violet for quantitation of cells showing intense cytoplasmic vacuolation (foamy cells). Sections were photographed using an Axiophot Zeiss light microscope. The number of foamy cells was quantitated in 12–15 sections per animal ($n=4–7$ animals per group) in Lamina IX ventral horn using a computerized image analysis system (Bioscan Optimas VI). In these experiments, data were reported for the following groups: (1) 2-month-old Wobbler mice; (2) 2-month + PROG; (3) 4-month-old Wobbler mice; (4) 4-month + PROG. Controls were not included since vacuolated cells were totally absent in normal animals.

2.4. Determination of serum PROG

Serum was obtained after blood collection from the heart. The content of PROG was determined using a Coat-A-Count Progesterone kit (Diagnostic Products, Los Angeles, USA). In spite that product specification stated that metabolites of PROG were not antibody bound, we found that 5α-dihydroprogesterone (5α-DHP) and 3α, 5α-tetrahydroprogesterone (3α, 5α-THP) were as potent as PROG to displace the iodinated ligand. Therefore, results were expressed as nanograms of circulating "total progestins" per milliliter of serum.

2.5. Statistical analysis

Data were expressed as mean ± S.E. To compare multiple group means, we used one-way ANOVA followed by the Newman–Keul's "post hoc" test.

3. Results

3.1. NADPHD histochemistry in motoneurons

In control mice, with or without PROG treatment, NADPHD-active motoneurons were scarce, amounting to less than 10 positive cells per mm^2 of ventral horn (Fig. 1, upper and lower graph). In contrast, ~30 NADPHD-positive cells per mm^2 were found in 2-month-old Wobbler mice, representing an eightfold increase over controls (Fig. 1 upper graph; $p<0.001$). This increase agrees with previous data [6,11]. Wobbler mice in this age group showed clinical signs typical of stage 2 of the disease, as described in the Materials and methods. In 2-month-old Wobbler mice, PROG pellet implantation lasting 2 1/2 weeks significantly reduced by more than 50% the number of NADPHD-positive neurons ($p<0.001$ vs. untreated Wobblers). Although the mean number of NADPHD-positive motoneurons in the Wobbler + PROG group remained higher than controls, they were not significantly different. Nevertheless, the increased NADPHD activity of Wobbler mice was only transient, since 4-month-old Wobbler mice showed similar levels to control mice of the same age group. In 4-month-old Wobbler mice, PROG treatment was ineffective towards NADPHD (Fig. 1, lower graph). All 4-month-old Wobbler mice showed characteristics of stage 3 or stabilization of the disease (Materials and methods). Fig. 2 shows the histomorphology of NADPHD-active motoneurons in the experimental group of 2-month-old mice. As clearly shown, NADPHD-positive motoneurons were absent or faintly stained in control (A) and control + PROG (B). These pictures contrasted with blue formazan reaction of perikaryon and cell processes in untreated Wobblers (C). Fig. 2D also shows the attenuation of NADPHD hyperexpression in Wobblers after 2 1/2 weeks of PROG treatment.

The MP experiment was carried out using 2-month-old control and Wobbler mice, due to the low number of NADPHD-active motoneurons in 4-month-old Wobbler animals. As shown in Fig. 3, the average number of NADPHD-active motoneurons was slightly higher in untreated Wobblers than in the Wobbler + MP group ($p<0.05$, ANOVA followed by a post hoc test) but both groups did not differ using the Student's t test. Therefore, the reduction of NADPHD staining of motoneurons obtained after MP treatment of Wobbler mice seemed of borderline significance compared to the pronounced PROG effect on this parameter.

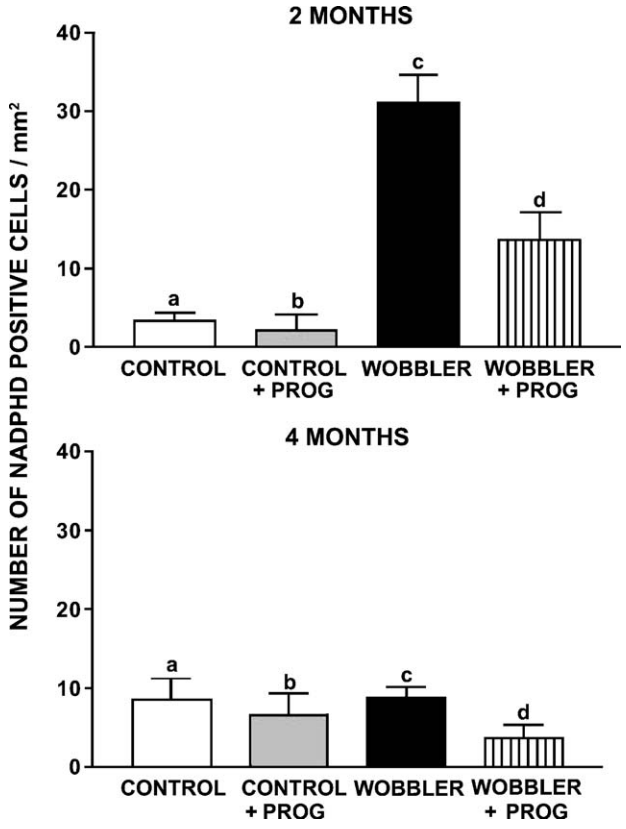

Fig. 1. Effects of progesterone (PROG) treatment on the number of NADPH-diaphorase active motoneurons in 2-month-old control and Wobbler mice (upper graph) and 4-month-old control and Wobbler mice (lower graph). Results represent the means ± S.E. of 12–15 sections per animal ($n = 4–7$ animals per group). Less than 10 NADPH-diaphorase active neurons per mm^2 were obtained in control mice of either age with or without PROG. PROG treatment was without effect in control mice (a vs. b, ns). The 2-month-old Wobbler group showed an eightfold increase in NADPH-diaphorase-positive motoneurons respect to control mice (upper graph, c vs. a, $p < 0.001$), which did not occur in the 4-month-old animals (lower graph, c vs. a, ns). PROG treatment significantly reduced NADPH-diaphorase positive neurons in the 2-month-old Wobbler mice (upper graph, c vs. d, $p < 0.001$) but not in the 4-month-old group (lower graph, c vs. d, ns). Statistical analysis by one-way ANOVA followed by the Newman–Keuls "post hoc" test.

3.2. NADPHD histochemistry in astrocytes

The number of NADPHD-active astrocytes was determined in the CST and VLF of the white matter from three animal groups: control, Wobbler and Wobbler + PROG-treated mice. NADPHD-active astrocytes were significantly increased in both CST and VLF of 2- and 4-month-old Wobbler mice compared to controls (Fig. 4 upper and lower graph). In 2-month-old Wobblers, PROG treatment reduced NADPHD-active astrocytes in the VLF ($p < 0.01$), but reduction in the CST did not reach significance. PROG was ineffective in 4-month-old Wobbler mice. In the MP experiment, steroid treatment of 2-month-old Wobbler mice did not change the number of NADPHD-active astrocytes in the CST or VLF (results not shown).

3.3. PROG effects on cell vacuolation and the percentage of NADPHD active cells

As already reported, cells with intense cytoplasmic vacuolation (foamy cells) are typical of Wobbler mice but are absent from control littermates [4,12,13,28]. Quantitative analysis demonstrated that the percentage of ventral horn foamy cells per square millimeter, with respect to the total ventral horn neuronal number, was higher in 2- than in 4-month-old Wobbler mice (Fig. 5). However, due to some overlapping, differences between age groups were not significant. However, PROG treatment reduced sixfold the percentage of vacuolated cells in 2-month-old Wobblers ($p < 0.05$) but was inactive in the older group. Hence, in motoneurons of 2-month-old Wobbler mice receiving PROG but not in the 4-month group identically treated, a reduction of NADPHD-active motoneurons was correlated with a decrease in vacuolated cells. Interestingly, 4-month-old Wobbler mice presented a reduction in the total number of ventral horn neurons per mm^2 compared to 2-month-old mice (97.4 ± 3.2 vs. 117.5 ± 4.4, $p < 0.01$), suggesting that part of the vacuolated neurons in the younger animals were destined to dye. Total neuronal number in 4-month-old Wobblers was kept at levels of 2-month-old Wobbler mice when PROG treatment was installed (114.3 ± 8.9, NS vs. 2-month-old mice).

Fig. 6 shows the typical motoneuron morphology in a control mouse (A), an intensely vacuolated cell in a Wobbler mouse (B) and two motoneurons in the Wobbler + PROG mice with different profiles: Although the motoneuron in (C) still presented a vacuolated cytoplasm, it was not as marked as that in the cell shown in (B). The cell shown in (D) revealed an almost normal appearance yet with fine vacuolation at the proximal end of the main neurite.

Since we already showed that there was a loss of motoneurons in 4 month-old Wobbler mice, it seemed important to determine the percentage of motoneurons that are NADPHD active in each aged group. Therefore, we quantitated the % NADPHD positive neurons/total motoneurons determined by Cresyl violet staining and expressed data per mm^2 of ventral horn. In the 2-month-old group, relevant data were ($n = 5–6$ mice per group): controls 2.2 ± 0.6; Wobblers 17.5 ± 2.3 ($p < 0.001$ vs. controls); and Wobbler + PROG-treated mice: 8.0 ± 2.5 ($p < 0.001$ vs. untreated Wobbler group). In the 4-month-old group, respective data were ($n = 5–7$ mice per group): controls 2.0 ± 0.5; Wobblers 5.6 ± 0.9 (NS vs. control group); and Wobbler + PROG-treated mice 2.1 ± 1.0 (NS vs. untreated Wobbler mice). Therefore, the considerable decrease in the % NADPHD positive cells in older animals suggested that this cell type was preferentially lost with advancing neurodegeneration.

3.4. Serum PROG levels

Levels of circulating progestins were low in 2-month-old (4.1 ± 0.6 ng/ml) and 4-month-old control male mice

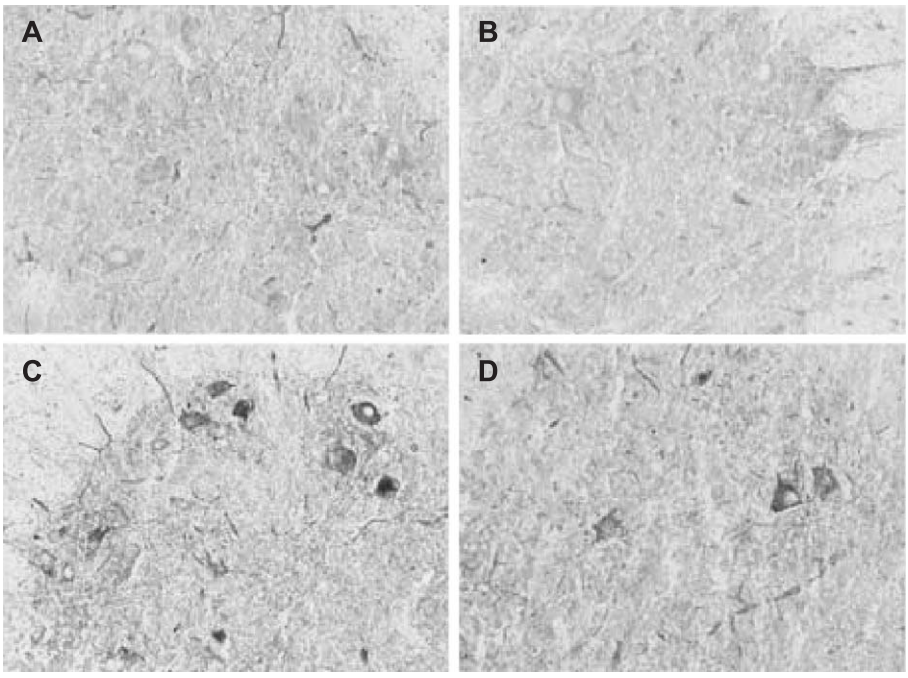

Fig. 2. Photomicrographs representing NADPH-diaphorase histochemistry in the spinal cord ventral horn of a control mouse (A), control + PROG (B), Wobbler mouse (C) and Wobbler treated with PROG (D) corresponding to the experimental group of 2-month-old mice. Hyperexpression of NADPH-diaphorase in Wobbler (C) is attenuated by PROG treatment (D). Magnification: 200×.

(6.4 ± 1.4). These levels were significantly elevated by PROG treatment (2-month: 29.6 ± 3; 4-month: 27.8 ± 2.1 ng /ml, $p < 0.001$ vs. age-matched controls). Untreated Wobbler mice also presented low levels of serum PROG, which were not significantly different from their respective controls (2-month: 11 ± 4.4; 4-month: 7.7 ± 1.4 ng /ml). After PROG administration, serum steroid levels were considerably elevated in Wobbler mice (2-month: 52 ± 3.1; 4-month: 75.4 ± 18.5 ng/ml, $p < 0.001$ vs. PROG-treated control mice). We already mentioned that the used antibody did not distinguish between the parent compound PROG and its reduced metabolites 5α-DHP and 3α, 5α-THP. Thus, serum data indicated that substantial levels of "progestins" remain in the circulation of control and Wobbler mice at the time of sacrifice. However, the disparity in "total progestin" levels between control and Wobbler mice suggested that there might be some difference in metabolism between the two types of mice. Indeed, preliminary data demonstrated a higher proportion of 5α- and 3α, 5α-reduced metabolites of progesterone in serum of Wobbler mice respect of control animals (55% vs. 6.2%), a difference that supports this possibility (unpublished data).

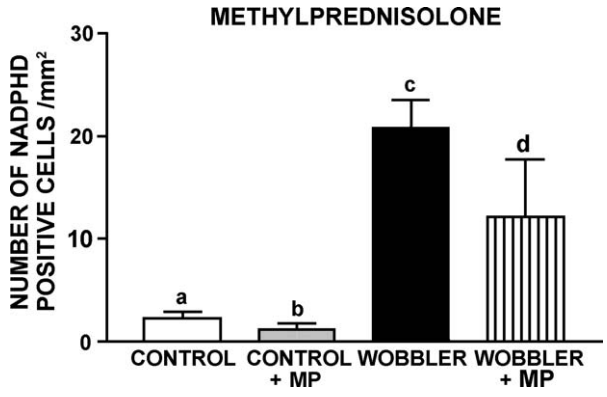

Fig. 3. Effects of methylprednisolone (MP) treatment on the number of NADPH-diaphorase active motoneurons in 2-month-old control and Wobbler mice. Results represent the means ± S.E. of 8–10 sections per animal ($n = 3–7$ animals per group). In agreement with data of Fig. 1, few NADPH-diaphorase positive neurons were observed in control mice, and MP did not affect this low number. In contrast, Wobbler mice presented a pronounced increase in NADPH-diaphorase positive neurons over control littermates (c vs. a, $p < 0.001$), which was slightly reduced by MP treatment (c vs. d, $p < 0.05$, ANOVA and post hoc test, p = NS by Student's t test).

4. Discussion

The Wobbler mouse has been considered as an excellent model for naturally occurring motoneuron diseases [4,8]. In this work, we first studied in Wobbler mice of two different stages the NADPHD/NOS activity in motoneurons and astrocytes and the effects of PROG and MP on this parameter. Our data indicated that: (a) the increased NADPHD activity and motoneuron vacuolation in Wobbler mice was age-dependent, because it occurred in 2- but not in 4-month-old Wobbler mice; (b) PROG down-regulated neuronal NADPHD and cell vacuolation exclusively in the 2-month-old Wobbler group; (c) although the increased NADPHD activity of astrocytes was present in both age

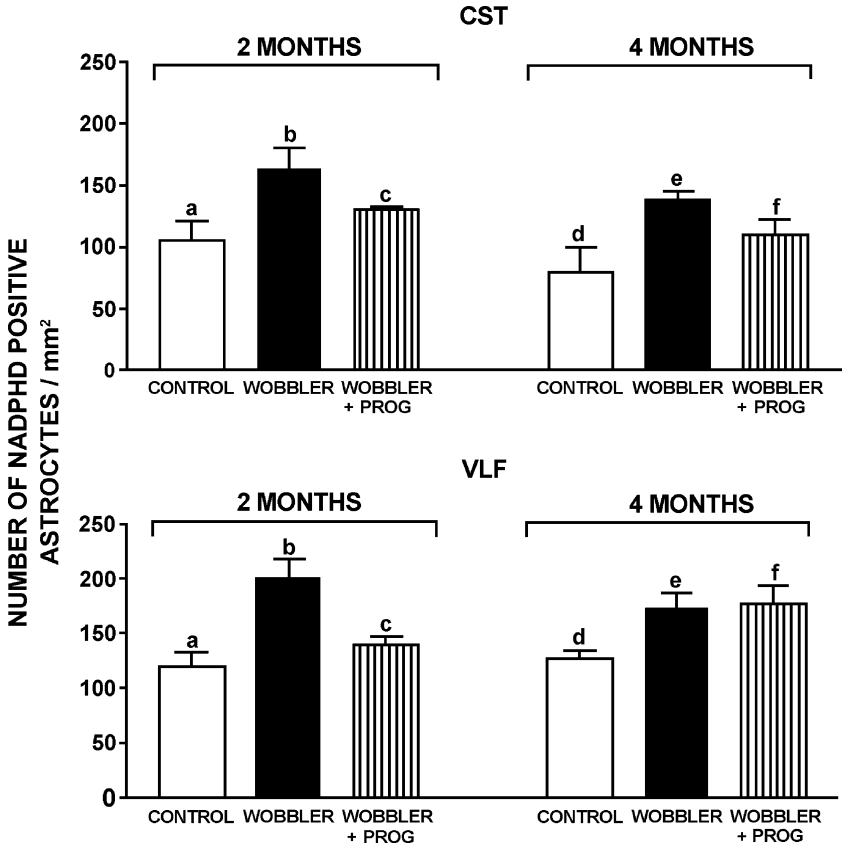

Fig. 4. Effects of PROG on the number of NADPH-diaphorase active glial cells in the white matter corticospinal tract (CST, upper graph) and ventrolateral funiculus (VLF, lower graph) in 2- and 4-month-old control animals (white columns), untreated Wobbler mice (dark columns) and Wobbler mice receiving PROG (vertical line columns). Results represent the means ± S.E. of 12–15 sections per animal ($n = 4–7$ animals per group). In 2-month-old Wobbler mice, NADPH-diaphorase-positive astrocytes were more abundant in CST (b vs. a, $p < 0.05$) and VLF (b vs. a, $p < 0.01$) than controls, while PROG treatment significantly reduced them in VLF only (c vs. b, $p < 0.01$). NADPH-diaphorase-active astrocytes in 4-month-old Wobblers were significantly increased in both white matter regions (e vs. d, $p < 0.05$), but were not modified by PROG. Statistical analysis as in the legend of Fig. 1.

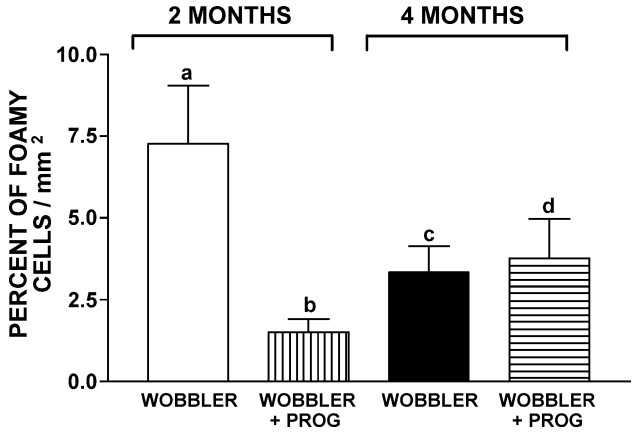

Fig. 5. Effects of PROG on the percent of vacuolated (foamy cells) in 2- and 4-month-old Wobbler mice. Data represent the means ± S.E. of 12–15 sections per animal ($n = 4–7$ animals per group). Groups studied comprised 2-month Wobbler mice (white column), 2-month Wobbler plus PROG (vertical line column), 4-month-old Wobbler (dark column) and 4-month-old Wobbler plus PROG (horizontal line column). Statistical significance: a vs. b, $p < 0.05$; c vs. d, ns. Vacuolated cells were absent in control mice.

groups, PROG reduced the enzyme in the 2-month group only; (d) the decrease in NADPHD-active motoneurons in 2-month-old Wobbler mice caused by PROG was mimicked by MP.

In agreement with previous reports [6,11,41], control mice presented low abundance of faintly stained NADPHD-active motoneurons. In contrast, motoneurons with high NADPHD activity in perikaryon and cell processes were detected in 2-month-old Wobblers. Since this change subsided by 4 months, it is likely that increased NADPHD characterized motoneurons committed to a degeneration pathway. This assumption was supported by counting the % NADPHD active motoneurons in each age group, which indicated that NADPHD staining was markedly attenuated in the 4-month-old Wobbler mice. In addition, motoneurons of 2-month-old Wobbler mice presented a pronounced cytoplasmic vacuolation, indicative of a cytoplasmic—Clarke's type II—cell death [5]. In this group, but not in the 4-month-old Wobbler group, PROG treatment significantly reduced NADPHD activity and the percentage of vacuo-

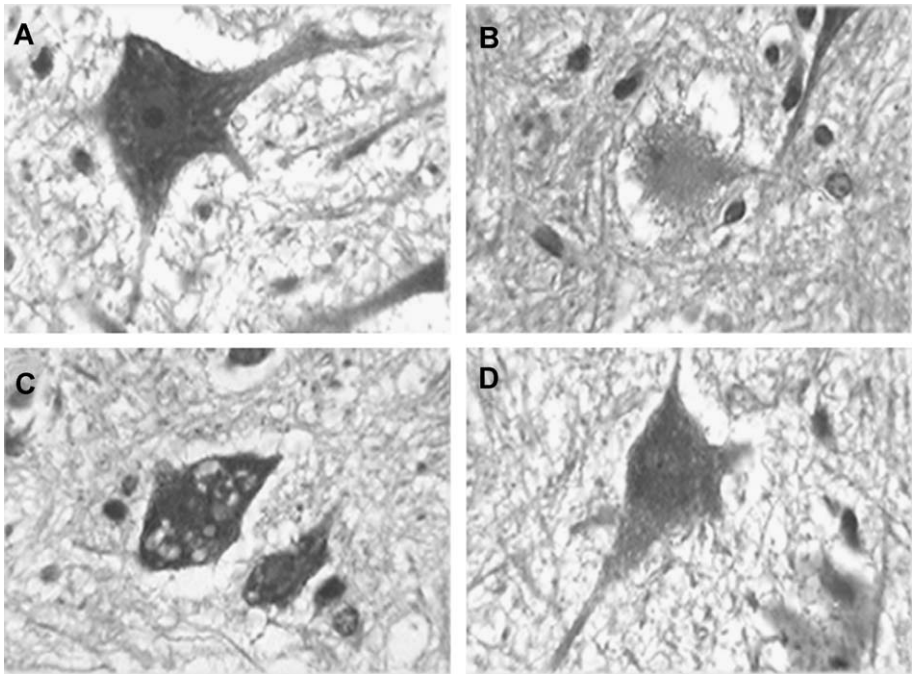

Fig. 6. Light microscopy photomicrographs of cervical spinal cords stained with Cresyl violet. The photographs correspond to motoneurons of control mouse (A), intensely vacuolated (foamy) cell in untreated Wobbler mouse (B) and two morphological types in Wobbler mice treated with PROG: cell with low number of small vacuoles (C) and cell of almost normal appearance (D). Magnification: 1000×.

lated cells. Therefore, these data suggest that PROG was blocking a factor operative at the initial stages of neurodegeneration. Then, it is possible that PROG prevented NO from reaching neurotoxic levels and protected endomembranes and mitochondria from oxidative damage occurring at the progressive or type 2 stages of the Wobbler disease.

Our data agrees with previous hypothesis [6,41] that NADPHD-positive neurons in Wobbler mice are likely degenerating neurons, in which increased NO production may be involved in a cell death program. Interestingly, the age period during which motoneurons mostly express NADPHD corresponds to the disease stage when animals present flexion of forelimb digits and slight tremor [43]. At this age period, vacuolated cells were at maximum, in contrast to the paucity of NADPHD-active and vacuolated motoneurons in 4-month-old mice. At this late period, the measured neuronal loss probably accounted for the intense tremor, ambulatory difficulty, curled wrists and marked muscle atrophy. Similarly, in the SOD1 transgenic mouse, another model of motoneuron disease, nNOS-positive anterior horn cells predominate in the early symptomatic period and were less prominent at the end stage of the disease [36]. Another source of excess NO are the astrocytes, which express the inducible form of the enzyme (iNOS). Diffusion of this astrocyte-derived NO damages neighboring motoneurons [39]. In the case of Wobbler mice, abundant NADPHD-positive astrocytes were located in the CST and VLF of both two and 4-month-old mice, in contrast to the biphasic NADPHD activity shown by motoneurons. This finding indicates that motoneurons were continuously exposed to an excess of NO, further implicating the importance of NO as a disruptor of neuronal function.

In this scenario, PROG reduction of NADPHD-active motoneurons and NADPHD stained astrocytes in the white matter VLF in 2-month-old Wr mice (progression stage) may represent an antioxidant effect. In support of this hypothesis, Roof et al. [31–33] consider that PROG beneficial effects against brain trauma and ischemia are based on antioxidant steroid properties, which stabilize plasma and intracellular membranes after intercalation between fatty acids component of phospholipids. This steroid ability could interfere with free radical attack on membranes [31,38]. Therefore, attenuation by PROG of oxygen-radical induced lipid peroxidation may explain the decreased vacuolation and partial recovery of mitochondrial morphology described in Wobbler mice receiving PROG [13,14]. It is likely that excess NO, by virtue of its capacity to block electron transport chain, is responsible for the release of free radicals from the mitochondria. Indeed, a recent report described mitochondrial dysfunction with impairment of complexes I, III and IV activity and decrease in states 3 and 4 respiration rates in Wobbler mice motor cortex and spinal cord [15,30,39]. The down-regulation of NADPHD in motoneurons obtained with MP, a synthetic glucocorticoid showing strong antioxidant properties [17,18], although of small magnitude, also suggests that part of PROG action may be due to blockade of damaging reactive oxygen species.

According to Beal [3], a common factor in neuropathology of degenerative diseases is the early occurrence of mitochondrial dysfunction. The assumption that pathological levels of NO, produced in neurons and/or glial cells of Wobbler mice, originated or potentiated neurodegeneration, is supported by the beneficial effects caused by pharmacological inhibition of NOS/NADPHD in Wobblers [11,20,41]. Thus, PROG and MP may be acting like the inhibitors 7-nitroindazole and BDNF used by these workers.

However, the antioxidant hypothesis may explain only part of PROG beneficial effects. A second mechanism relays on the ability of PROG and its reduced derivatives to activate GABAa receptors, while inhibiting NMDA glutamate receptors [34]. Determination of serum steroids by RIA demonstrated that high levels of circulating progestins were present in Wobbler mice. Preliminary data showed that high levels of the 5α- and 3α, 5α-reduced metabolites of PROG are present in plasma and spinal cord of Wobbler mice receiving PROG treatment (to be published). These metabolites are as potent as PROG in recovering myelination of aged peripheral nerves [2]. However, the actual importance of modulation of neurotransmitter receptors in PROG effects in Wobbler mice remains to be elucidated. In addition to the considered mechanisms, it is possible that PROG was acting through a genomic mechanism, since a constitutive form of the PR is expressed by rodent spinal cord motoneurons and astrocytes [25]. Therefore, the possibility exists that PROG effects on NADPHD were transcriptionally mediated in the degenerating tissue. Evidently, further experimental data are needed to elucidate the multiplicity of PROG effects in neurodegeneration models.

Although the current data showed that PROG treatment reduced NADPHD staining in Wobbler mice in the progressive stage, the treatment was given for a short time, which made difficult to evaluate if a significant neuroprotection occurred at a later time point. However, we have gathered evidences that this may be the case. First, a more prolonged course of steroid treatment concluded that PROG was able to ameliorate muscle atrophy. Thus, although weight of the biceps brachii was markedly reduced in 4-month-old untreated Wobbler mice respect of age-matched control mice, it increased by 1.7-fold after 60 days of PROG treatment (unpublished data). Second, in a previous report, we followed up the response of the α_3 mRNA for the Na, K-ATPase 60 days after the steroid was implanted into 2-month-old Wobbler mice. In that study, the mRNA level of the ion transporter was reduced in untreated Wobblers compared to control mice, but it increased to near normal levels in the PROG-treated Wobbler group, suggesting a long lasting neuroprotective effect [13]. These demonstrations, in addition to present results, indicate that inhibition of NO-mediated degeneration by steroid hormones may offer therapeutic possibilities for diseases in which oxidative attack play an important pathogenic role.

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