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Progesterone Neuroprotection in the Wobbler Mouse, a Genetic Model of Spinal Cord Motor Neuron Disease

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Motor neuron degeneration characterizes the spinal cord of patients with amyotrophic lateral sclerosis and the Wobbler mouse mutant. Considering that progesterone (PROG) provides neuroprotection in experimental ischemia and injury, its potential role in neurodegeneration was studied in the murine model. Two-month-old symptomatic Wobbler mice were left untreated or received sc a 20-mg PROG implant for 15 days. Both light and electron microscopy of Wobbler mice spinal cord showed severely affected motor neurons with profuse cytoplasmic vacuolation of the endoplasmic reticulum and/or Golgi apparatus and ruptured mitochondria with damaged cristae, a profile indicative of a type II cytoplasmic form of cell death. In contrast to untreated mice, neuropathology was less severe in Wobbler mice receiving PROG; including a reduction of vacuolation and of the number of vacuolated cells and better conservation of the mitochondrial ultrastructure. In biochemical studies, we determined the mRNA for the α3 subunit of Na,K-ATPase, a neuronal enzyme controlling ion fluxes, neurotransmission, membrane potential, and nutrient uptake. In untreated Wobbler mice, mRNA levels in motor neurons were reduced by half compared to controls, whereas PROG treatment of Wobbler mice restored the expression of α3 subunit Na,K-ATPase mRNA. Therefore, PROG was able to rescue motor neurons from degeneration, based on recovery of histopathological abnormalities and of mRNA levels of the sodium pump. However, because the gene mutation in Wobbler mice is still unknown, further studies are needed to unveil the action of PROG and the mechanism of neuronal death in this genetic model of neurodegeneration. © 2003 Elsevier Science (USA)

Key Words: **neuroprotection; progesterone; spinal cord; neurodegeneration; Wobbler mice; amyotrophic lateral sclerosis.**

INTRODUCTION

Animal models with genetic defects remain useful tools to explore therapeutic alternatives for human neurodegenerative diseases. In the Wobbler mouse, a mutation of autosomic recessive expression (wr) produces motor neuron degeneration in the spinal cord and brain stem (Duchen and Strick, 1968). Although the wr gene has not been identified, it maps to chromosome 11 (Kaupmann *et al.*, 1992) close to the glutamine synthase gene (Des Portes *et al.*, 1994). Due to clinical symptoms and prevalent neuropathology, Wobblers become useful models of motor neuron disease including amyotrophic lateral sclerosis (ALS) and infantile spinal muscular atrophy (Werdenig–Hoff-

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man disease) (reviewed in Cudkowicz and Brown, 1998; Price *et al.*, 1994).

In homozygous animals (wr\wr) the first manifestations of the disease are observed at 2–3 weeks of age, consisting of progressive muscle weakness, tremor, and ambulatory difficulty, followed by muscle atrophy, forelimb flexion, and extension of the rear limbs (Duchen and Strick, 1968). Molecular biology studies in Wobbler mice spinal cord showed increased expression of genes involved in trophism and differentiation during embryonic life, hyperexpression of the growth-associated protein GAP-43, and aggregation of medium neurofilaments (Junier *et al.*, 1994, 1998; Popper *et al.*, 1997; Pernas-Alonso *et al.*, 2001). Some of these changes are early events, which appear before the development of neurodegeneration.

Morphologically, anterior horn cells of Wobbler mice undergo a dramatic perikaryal vacuolar degeneration (Mitsumoto and Bradley, 1982), in addition to swelling of α and γ motor neurons, interneurons, and Renshaw cells (Junier *et al.*, 1994; Dockery *et al.*, 1997). This change is accompanied by pronounced astrocytosis (González Deniselle *et al.*, 2001) and increased density of activated microglia (Boillee *et al.*, 2001). Whereas motor neurons in 3- to 4-week-old Wobbler mice are TUNEL negative (Popper *et al.*, 1997; Pernas-Alonso *et al.*, 2001), a transient massive DNA fragmentation of neurons and glial cells was reported by Blondet *et al.* (2001) before the manifestation of clinical symptoms. Therefore, while apoptosis may cause cell death in the early stages, motor neuron pathology of symptomatic mice resembled the type II or cytoplasmic form of cell death (Clarke, 1990). Extensive cytoplasmic vacuolar degeneration also exists in SOD1 transgenic mice, a generated model of ALS in which free-radical damage of cell membranes plays a preponderant role (Gurney *et al.*, 1996; Gurney, 2000; Kong and Zu, 1998). In Wobbler mice, participation of oxidative stress is supported by abnormalities of mitochondrial function (Guang-Ping *et al.*, 2001) and the clinical, biochemical, and morphological improvement caused by treatment with antioxidants, steroids, or nitric oxide inhibitors (Ikeda *et al.*, 1998, 2000; Henderson *et al.*, 1996; González Deniselle *et al.*, 2001). At least one of the brain-derived neurotrophic factor's protective effects in Wobbler mice may be mediated by the reduction of reactive oxygen species (Tsuzaka *et al.*, 2001). Thus, both oxidative stress and apoptosis acting in a different time sequence may cause cell death in motor neuron degeneration models (Li *et al.*, 2000; Blondet *et al.*, 2001).

Several treatment protocols are available to prevent or delay neurodegeneration. Novel agents include gonadal steroid hormones, which control gene expression and multiple regulatory pathways in the brain and spinal cord (Azcoitia *et al.*, 2001; De Nicola, 1993; Mc Ewen, 1999). In the case of estrogens, neuroprotective and neurotrophic effects are well documented using *in vitro* preparations, whole animal experiments, and patients with Alzheimer's disease (Brinton, 2001; Henderson, 1997; Mc Ewen, 1999; Toran-Allerand, 2000). However, some questions remain, since in subsequent work, Henderson (1997) reported that short-term estrogen therapy did not prevent cognitive decline of women with mild to moderate Alzheimer's disease.

Estradiol combined with progesterone (PROG) showed neuroprotective effects in an animal model of neurodegeneration (Vongher and Frye, 1999). However, effects of single PROG treatment were reported mostly in injury models (Schumacher *et al.*, 2000). For instance, PROG stimulated myelinogenesis in injured peripheral nerves (Koenig *et al.*, 1995; Desarnaud *et al.*, 1998) and prevented neuronal loss following brain injury, ischemia, and edema (Chen *et al.*, 1999; Roof and Hall, 2000; Stein and Fulop, 1998). In the spinal cord, treatment of rats with PROG increased motor neuron survival after axotomy or injury (Yu, 1989; Thomas *et al.*, 1999), protected cultured neurons against glutamate toxicity (Ogata *et al.*, 1993), and normalized several defective parameters of injured motor neurons (Labombarda *et al.*, 2001). Although the spinal cord PROG receptor may play a role (Labombarda *et al.*, 2000b), antioxidant and other membrane-based mechanisms may be also significant components of PROG effects (Roof and Hall, 2000). However, it should be mentioned that evidences of PROG neuroprotection are counterbalanced by one report showing exacerbation of subcortical stroke injury due to chronic progesterone treatment of ovariectomized rats (Murphy *et al.*, 2000).

The beneficial effects of PROG in injury models encouraged the use of Wobbler mice to explore whether PROG played a similar role in neurodegeneration. On these premises, the following objectives were pursued: first, to establish in 2-month-old, clinically affected Wobbler mice the prevalent motor neuron pathology at the light and electron microscopy levels; second, to explore the function of motor neurons by measuring the mRNA for the α3 subunit of the Na,K-ATPase. This enzyme showed PROG sensitivity in the spinal cord (Labombarda *et al.*, 2001) and its pivotal role in neurotransmission, nutrient uptake, and maintenance of membrane potential is well established (Lees, 1991; Stahl, 1986); third, to analyze whether PROG treatment rescued motor neurons from degeneration, based on recovery of morphology

and mRNA levels of the Na,K-ATPase. Our data constitute the first evidence that PROG retards neurodegeneration in Wobbler mice spinal cord and, in this context, it may be potentially valuable for the treatment of human neurodegenerative diseases.

MATERIALS AND METHODS

Experimental Animals

Heterozygous NFR/wr male and female breeder mice were obtained from The Animal Center, National Institutes of Health (Bethesda, MD, courtesy of Dr. Carl Hanson) and mated in our institute. Animals were housed in group cages containing 2–3 Wobbler and 1 control mouse under conditions of controlled humidity and temperature (22°C), with lights on from 0700 h to1900 h. This social interaction prolonged the life span and health status of Wobbler mice (M.-P. Junier, personal communication). Homozygous Wobbler mice (*wr/wr*) were 2 months old when used, representing stages 3–4 of the disease according to the criteria of Yung *et al.* (1982). All affected animals showed reduced body weight; and clinically they presented tremor, ambulatory difficulty, flexion of proximal limbs, distal limb extension, positive clasp knife reflex response, and diminished muscle strength. Routine histology showed motor neuron loss and astrogliosis of the spinal cord (González Deniselle *et al.*, 2001). A group of control and Wobbler mice received under the skin of the neck a 20-mg pellet of PROG under light ether anesthesia, and animals were used 15 days after steroid pellet implantation. The efficiency of this treatment was checked by measuring PROG levels by gas chromatography/mass spectrometry (Liere *et al.*, 2000), which demonstrated that PROG pellet implantation resulted in 10-fold higher PROG levels in the cervical spinal cord compared to untreated Wobbler mice (unpublished results).

Clinical effectiveness of PROG neuroprotection was tested in two ways. First, in a grip strength test (Coulpier *et al.*, 1996), animals were placed on a vertical grid and the time spent on the grid was recorded. Second, survival time after PROG pellet implantation was measured from day 0 to day 15. Differences in grip strength and survival time were analyzed by two-tailed Student's *t* test.

In Situ Hybridization for the α3 Subunit of Na,K-ATPase

Anesthetized animals were perfused transcardially with 0.9% NaCl prepared in diethylpirocarbonate-treated water, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. Spinal cords were immediately removed following dorsal laminectomy, postfixed 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. *In situ* hybridization (ISH) for the α3 subunit Na,K-ATPase mRNA was carried out following previously published protocols (González *et al.*, 1996; González Deniselle *et al.*, 1999a). The coding sequence for the α3 subunit of the Na,K-ATPase subunit was 5′ CGGGAAGAGCGGCGGACAGGCTGGTGAGCGGTGGCCGCAGA 3′. This oligonucleotide (Oligos Etc., Inc., Wilsonville, OR) produced strong hybridization signals in ventral horn motoneurons without labeling of glial cells. The probe was end labeled with [^{35}S]ATP using the enzyme deoxynucleotidyl terminal transferase (Boehringer Mannheim, Germany).

Cryostat sections obtained at the cervical level were fixed in 2% paraformaldehyde, washed in 0.5× SSC (1× SSC: 0.15 M sodium chloride/0.015 M sodium citrate buffer, pH 7.2), and acetylated with acetic anhydride. Hybridization proceeded overnight at 40°C with 9×10^6 cpm ^{35} S-labeled probe/ml in a medium containing 0.02% Ficoll 400, 0.02% polyvinylpirrolidone, 0.02% BSA, 50% formamide, 3× SSC buffer, 10 mM dithiothreitol, 0.1 mg/ml salmon sperm DNA, 1 mM EDTA, 4 μg/ml heparin, 0.4 mg/ml tRNA, and 10% dextran sulfate. At the end of hybridization, sections were washed several times in SSC, dried, dipped into Kodak NTB-2 emulsion, and exposed in the dark for 2 weeks. Afterward, sections were developed with Dektol (Kodak, 1:2 dilution with water), fixed, counterstained with cresyl violet, and coverslipped with Permount. As controls for nonspecific hybridization, the signal of the ^{35}S-labeled probe was competed with a 20-fold excess of unlabeled oligonucleotide. Under this condition, the signal was drastically reduced to background levels. Also, a specific signal was absent in tissue preincubated with RNAse (20 μg/ml, 30 min at 37°C) before ISH.

For analysis of ISH, the number of grains per cell in motor neurons was calculated after background subtraction. Quantitative grain counting was performed by computer-assisted image analysis equipped with a video camera (Bioscan Optimas II). The area of individual neurons and grain density (number of grains per unit area of soma) were measured and results expressed as the mean ± SEM. Data from about 45 cells per animal corresponding to six sections ($n = 6$ mice per group) were combined to give an animal mean, and the animals were used as independent variables. Differences in the number of grains/100 μm^2 were determined by one-

way ANOVA, followed by post hoc comparisons with the Newman–Keuls test.

Light and Electron Microscopy

Wobbler mice treated with PROG, those without treatment, and control animals were anesthetized and perfused transcardially with a solution containing 4% paraformaldehyde plus 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Cervical spinal cords were removed and small blocks of tissue were obtained by cutting transverse sections of 2- to 3-mm maximum length. Blocks were immersed for 2 h in the same fixative. After being washed overnight in 0.1 M sodium phosphate buffer, tissue blocks containing the ventral horns were postfixed in 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4, for 1 h and stained with 1% uranyl acetate. Afterward, tissue blocks were dehydrated and flat-embedded in Durcupan (Fluka Chemic AG, Sweden). Semithin sections (0.8–1 μm) were stained with toluidine blue for light microscopy (LM) observations and quantitation of cells showing cytoplasmic vacuolation ("foamy cells"). Semithin sections were photographed using an Axiophot Zeiss light microscope. The number of foamy cells was quantified in 8–10 sections per animal ($n = 5$ animals per group) in Lamina IX ventral horn using a computerized image analysis system (Bioscan Optimas II). For statistical analysis, we employed one-way ANOVA followed by the Newman–Keuls *post hoc* test. For electron microscopy (EM), ultrathin sections (60–70 nm) were obtained with a Reichert ultramicrotome (Vienna, Austria) from ventral horns. Sections were stained with lead citrate, examined at 4400× and 20,000× magnifications, and photographed using a Zeiss 109 electron microscope.

RESULTS

In Situ Hybridization of α3 Subunit Na.K-ATPase mRNA

The effects of PROG treatment on the α3 subunit mRNA for the NA,K-ATPase were investigated in Wobbler mice subjected to 15-day steroid exposure as well as in untreated Wobbler mice and age-matched controls. In this experiment, neurons measuring >300 μm^2 localized in Lamina IX were considered α motor neurons based on anatomical location and size. As expected from previous results, control motor neurons showed a substantial grain density representing oligonucleotide probe hybridized to the neuronal α3 sub-

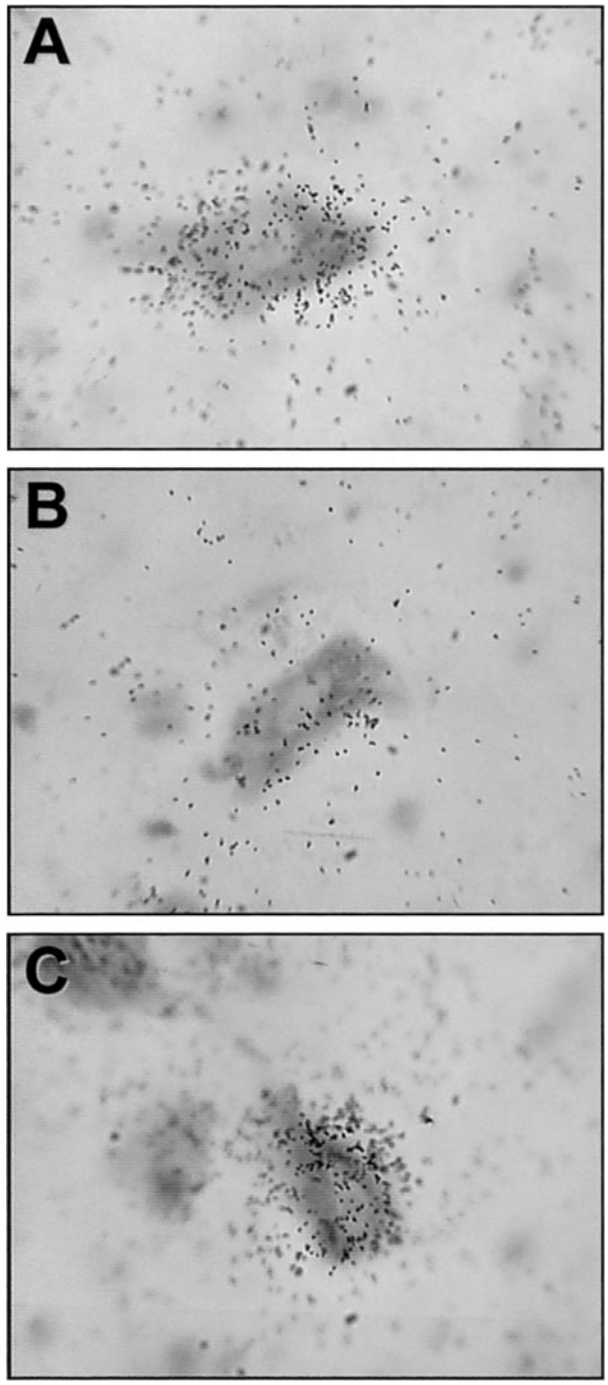


FIG. 1. *In situ* hybridization of the α3 subunit mRNA of the Na,K-ATPase in large motor neurons of Lamina IX of the spinal cord ventral horn. Light microscopy observations show abundant grain density in control mouse motor neuron (A), a paucity of grains in untreated Wobbler (B), and restored levels of mRNA in a motor neuron of a Wobbler mouse receiving progesterone for 15 days (C). Original magnification: 1000×.

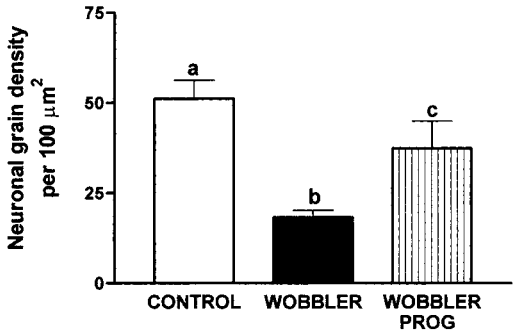


FIG. 2. Computerized image analysis followed by statistical comparison (one-way ANOVA and Newman–Keuls post hoc test) of *in situ* hybridization for the α3 subunit mRNA levels of the Na,K-ATPase. Grain density is significantly greater in control mice motor neurons than in untreated Wobblers (a vs b, $P < 0.001$). In Wobbler mice treated with progesterone (PROG) during 15 days levels are higher than in untreated Wobblers (b vs c, $P < 0.05$) but nonsignificantly different from controls. About 45 motor neurons were counted per animal, corresponding to six spinal cord sections at the cervical level. Data represent the mean ± SE of 6 animals per experimental group.

unit of Na,K-ATPase mRNA (Fig. 1A). This pattern contrasted with a severe grain depletion in motor neurons from Wobbler mice (Fig. 1B). Figure 1C also shows that after 15 days of PROG exposure, the hybridization signal in Wobbler + PROG mice was higher than that in untreated Wobblers. Quantitation of ISH data further confirmed that untreated Wobbler mice showed a dramatic 3.6-fold reduction in expression of the α3 subunit mRNA for the Na,K-ATPase when compared to control animals (Fig. 2; $P < 0.001$). In Wobbler mice treated with PROG, grain density doubled with respect to untreated Wobblers ($P < 0.05$), whereas there were nonsignificantly different from controls. This result indicated that PROG treatment was able to restore the severe reduction of α3 Na,K-ATPase mRNA expression shown by Wobbler mice in ventral horn motor neurons.

In principle, the PROG treatment was given for 2 weeks, which seemed a short time to evaluate significant neuroprotection. However, in one experiment the α3 mRNA for the Na,K-ATPase was determined 60 days after steroid implant. In this case, the number of grains per square micrometer in untreated Wobbler mice (22.0 ± 1) was still reduced compared to control animals (40.5 ± 6.7; $P < 0.01$, $n = 6$ animals per group). However, grain density was significantly recovered in the PROG-treated Wobbler group (34.1 ± 2.7, $n = 6$; $P < 0.05$) with respect to untreated Wobbler mice, suggesting a long-lasting neuroprotective effect.

Light and Electron Microscopy

In toluidine-blue-stained semithin sections of the ventral horn observed at low power, control mice motor neurons showed a normal appearance and lack of vacuolation (Figs. 3A and 3B). In contrast, severely degenerating motor neurons of untreated Wobbler mice showed swollen soma and an important cytoplasmic vacuolar degeneration. Qualitatively, although vacuolated α motor neurons were detected in both untreated Wobbler and Wobbler mice receiving PROG, severely affected neurons predominated in the untreated Wobbler group (Figs. 3C and 3D). In PROG-treated Wobblers, neurons were less vacuolated (Fig. 3E) and some even displayed a normal morphology (Fig. 3F). The effect of PROG was quantitatively evaluated using computerized image analysis, which demonstrated that the total number of vacuolated (foamy) neurons per square millimeter found in untreated Wobblers (180.5 ± 22/mm^2) was significantly reduced in the group of Wobbler mice receiving PROG (119 ± 8/mm^2, $P < 0.05$) (Fig. 4). Furthermore, while in untreated Wobbler mice foamy cells represented 25.8 ± 0.93% of the total number of ventral horn neurons, this number was reduced to 12.9 ± 0.93% in Wobbler mice receiving PROG ($n = 5$ animals per group, five sections analyzed per animal; $P < 0.001$).

EM also disclosed important morphological differences between control and Wobbler mice. Low-power EM (4400×) of control mice showed nuclear, nucleolar, and cytoplasmic ultrastructure typical of normal motor neurons (Fig. 5A, top). In contrast, severely affected neurons, which may represent an advanced degeneration stage, predominated in untreated Wobbler mice (Fig. 5B, middle). In this case, motor neurons were characterized by a normal nucleus, although the nuclear membrane showed an irregular festoon-like contour. Membrane breaking points, as reported in central nervous system apoptosis (Dikranian *et al.*, 2001), were not evident (Fig. 5B). In untreated Wobbler mice, large vacuoles probably derived from degenerating cysternae of smooth and rough endoplasmic reticulum, Golgi apparatus, or degenerating mitochondria occupied most of the cytoplasm and concentrated heavily around the nucleus, even altering nuclear shape (Fig. 5B). In Wobbler mice receiving PROG, neuronal degeneration was very moderate, as shown in the typical foamy cell of Fig. 5C (bottom). In the perinuclear area of this cell, few vacuolae remained and rough endoplasmic reticulum was largely

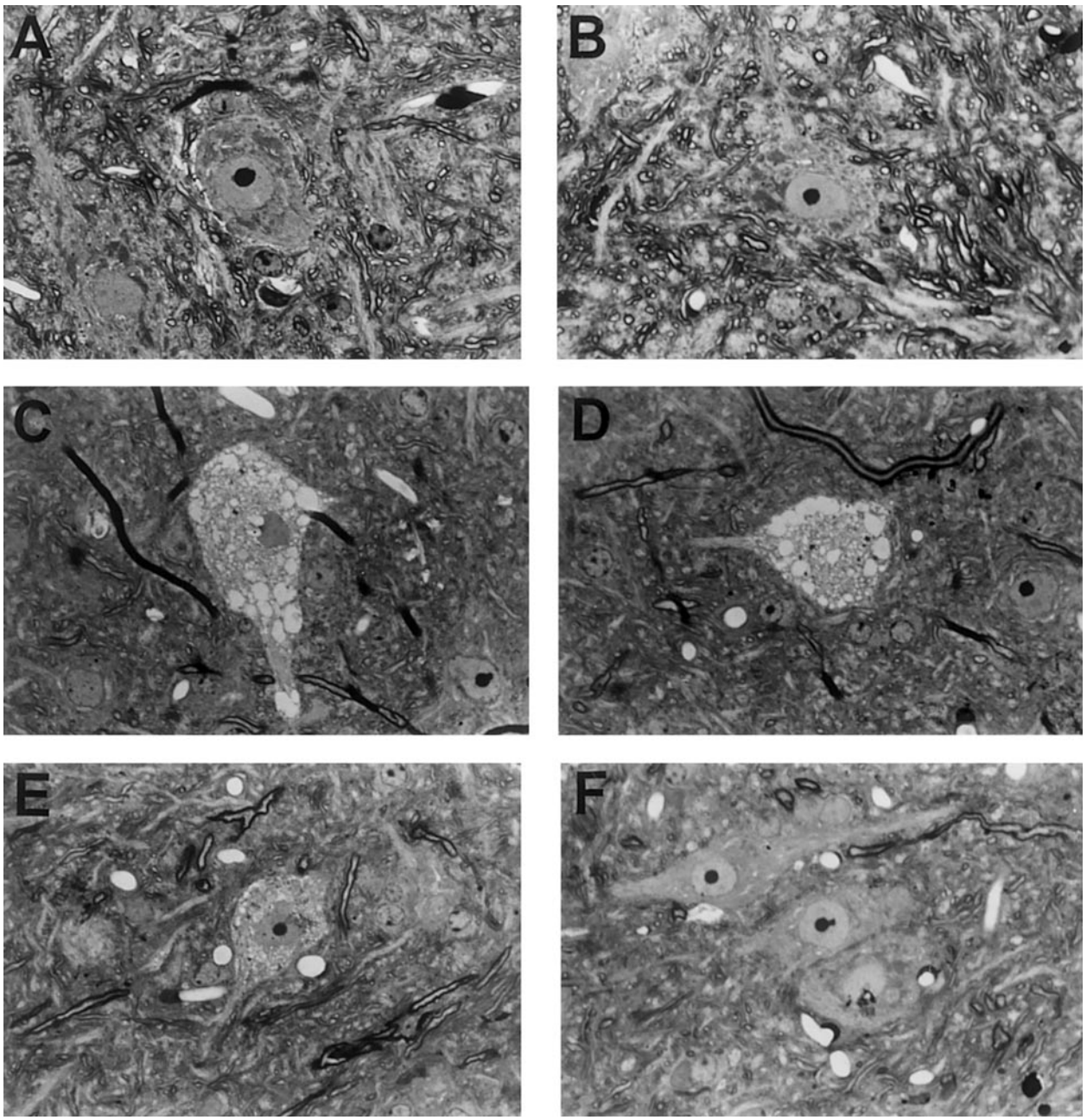


FIG. 3. Light microscopy observations of semithin sections of cervical spinal cord Lamina IX stained with toluidine blue. Photomicrographs show two control motor neurons (A, B), intensely vacuolated (foamy) cells in untreated Wobbler (C, D), and two morphological types in Wobbler mice treated with PROG: cell with a low number of small vacuoles (E) and a cell of normal appearance (F). Original magnification: 1000×.

preserved. The shape of the nuclear membrane was partly restored and some perinuclear mitochondria regained a normal appearance.

Higher magnification (20,000×) allowed a better appraisal of mitochondrial changes in the experimental animals. As opposed to normal mitochondria (Fig. 6A, top), the severely affected neurons of untreated Wobbler mice presented vacuolation and a disorganized mitochondrial ultrastructure, including edematous matrix, cristolysis, and loss of membrane integrity near the poles. Phagosomes (dark bodies) were also seen (Fig. 6B, middle). PROG treatment of Wobbler mice reverted in part these parameters, in that some mitochondria reassumed a normal ultrastructure (Fig.

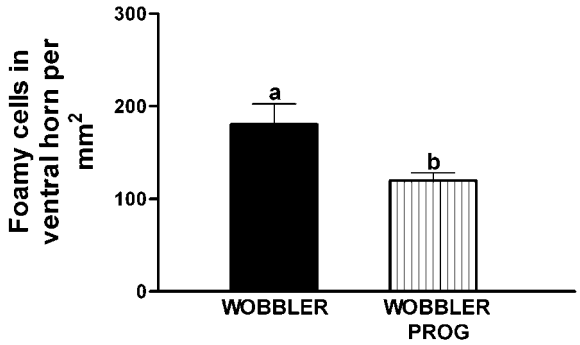


FIG. 4. Number of vacuolated (foamy cells) per square millimeter in untreated Wobbler mice (dark column) and Wobbler mice receiving PROG during 15 days (vertically lined column) (a vs b, $P < 0.05$, Student's *t* test). Vacuolated cells are absent in control mice. Figures represent the average counting of 8–10 sections per animal, five animals per group.

6C, bottom). Therefore, LM and EM observations indicated that PROG attenuated the cytoplasmic vacuolization of degenerating motor neurons of Wobbler mice spinal cord, reduced the density of vacuolized cells, and partly restored mitochondrial morphology. Also, typical features of apoptosis such as rupture of nuclear membrane with mixing of nuclear and cytoplasmic components, plasma membrane blebbing, or apoptotic bodies were missing from the majority of motor neurons of either untreated or PROG-treated symptomatic Wobbler mice. Occasionally, a cell displaying chromatin clumping was observed in Wobbler mice aged 5 months and older (González Deniselle *et al.*, unpublished).

Clinical Testing of PROG Neuroprotection

In a first experiment, we studied whether PROG slowed the deterioration of muscle function at the time that it prevented motoneuron degeneration. In a grip strength test (Coulpier *et al.*, 1996) untreated Wobbler mice remained fewer than 5 s in the grid, whereas Wobbler mice receiving PROG for 15 days tripled the time spent on the grid (Fig. 7A; $P < 0.05$).

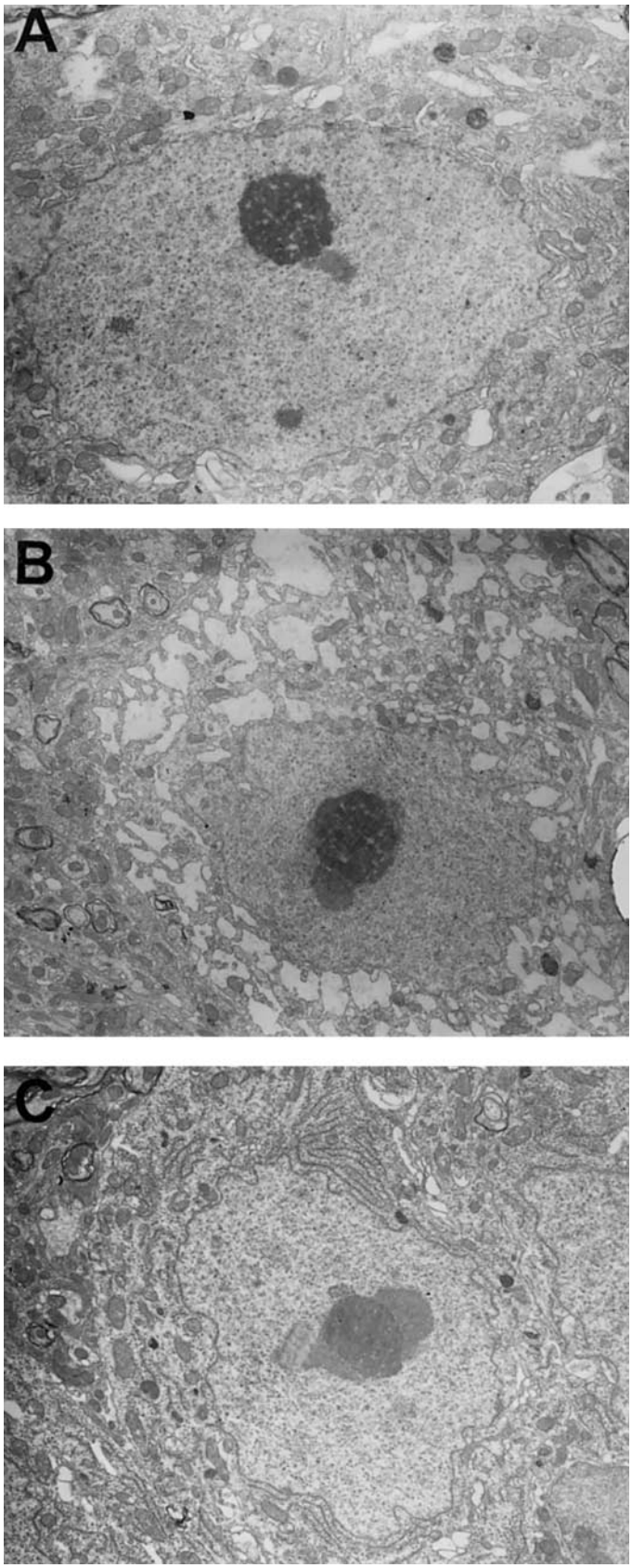
We also studied whether PROG treatment prolonged survival of the mutant mice. Figure 7B shows that the percentage of survival time in both untreated and PROG-treated Wobbler mice was identical for the first 7 days, after which PROG-receiving mice deviated from the untreated group. On day 15, a significant difference ($P < 0.03$) was measured due to increased survival of Wobblers receiving PROG compared to the untreated group.

DISCUSSION

The present experiments investigated PROG effects in a neurodegeneration model, based on previous evidences for PROG protection in CNS ischemia and trauma. Our data indicated that in addition to restoration of motoneuron morphology and at least one biochemical marker of motoneuron function (Na,K-ATPase), PROG also increased muscle strength and prolonged survival time of Wobbler mice. Thus, effects on these parameters supported the neuroprotective action of PROG treatment.

Employing LM and EM, the prevalent neuropathology found in symptomatic Wobbler mice consisted of motor neurons with cytoplasmic vacuolation and damaged mitochondria but with relative preservation of the nuclear ultrastructure. Previous observations using Wobbler mice at stages 3–4 of the disease described similar changes (Coulpier *et al.*, 1996; Dockery *et al.*, 1997; Mitsumoto and Bradley, 1982; Popper *et al.*, 1997; Yung *et al.*, 1982). At this stage of the disease, signs of overt apoptosis such as pyknotic nuclei, nuclear membrane fracture, apoptotic bodies, and plasma membrane blebbing were absent. In 2-month-old Wobbler mice chromatin clumping was not observed, although it was found in some older mice (results not shown). In contrast, massive DNA fragmentation reportedly occurred in presymptomatic Wobbler mice (Blondet *et al.*, 2001). However, it should be taken into account that in 2-month-old Wobbler mice the disease is poorly evolutive (Baulac *et al.*, 1983; Pollin *et al.*, 1990). Experiments using younger animals might yield useful information regarding PROG effects on degenerating neurons, including those undergoing apoptosis (Blondet *et al.*, 2001).

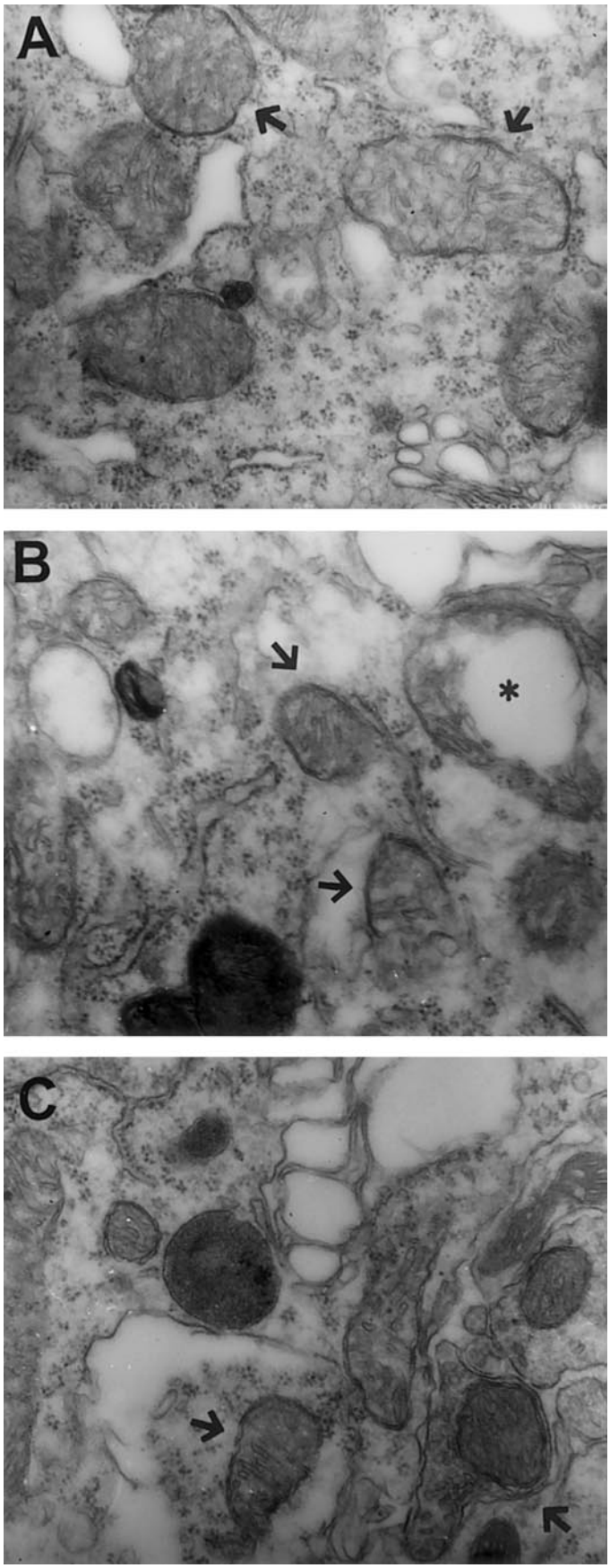
When morphological features of different types of cell death, i.e., apoptosis, necrosis, and cytoplasmic cell death, were compared to the present findings, we concluded that neurodegeneration in symptomatic Wobbler mouse resembled the last form, also known as type II or the autolytic type (Chu-Wang and Oppenheimer, 1978; Clarke, 1990). In this process, dilatation of smooth endoplasmic reticulum cysternae and Golgi system led to their autolytic breakdown, as already shown in clinically affected Wobbler mice (Popper *et al.*, 1997). Another important ultrastructural observation in untreated Wobbler mice was the presence of vacuolized mitochondria, with cristolisis, edema, and lack of outer membrane integrity. At the functional level, a mitochondrial respiratory chain dysfunction was observed in Wobbler mice (Guang-Ping



et al., 2001). This group reported that superoxide production, a leading cause of lipid peroxidation, may cause motor neuron death. Besides, lipid peroxidation was proposed as a leading causative factor for the cytoplasmic and mitochondrial changes in neurodegeneration (Mattson, 1998). A cytoplasmic form of cell death may also play a role in the mutant SOD1 transgenic model (G93A and G37R mutations), in which a toxic gain of function of the mutated enzyme led to oxygen-radical induced lipid peroxidation of intracellular membranes and mitochondrial abnormalities (Hall *et al.*, 1998; Kong and Zu, 1998; Mourelatos *et al.*, 1996). Interestingly, spinal cord neuropathology of Wobbler mice bears striking similarities to that of SOD1 transgenics, which also showed extensive vacuolation of mitochondria (Gurney, 2000; Wong *et al.*, 1995; Kong and Zu, 1998). Similarly, while apoptotic changes and chromatin clumping were reported by some workers in SOD1 transgenics and Wobbler mice (Blondet *et al.*, 2001; Spooren and Hengerer, 2000; Morrison *et al.*, 1998), others sustained that motor neuron death occurs primarily by massive vacuolation in the absence of apoptosis (Migheli *et al.*, 1999; Popper *et al.*, 1997). Thus, it is likely that at least two mechanisms—apoptosis and cytoplasmic vacuolization—caused motor neuron degeneration and death in the genetic and transgenic models of ALS. However, in the case of mitochondrial alterations, they may also be caused by secondary consequences, in which blood supply is probably lessened by muscle atrophy.

Therefore, it is possible that free radicals produced in neurons or glial cells originated and / or exacerbated neurodegeneration of untreated Wobbler mice. In these animals, blockage of free radical damage to motor neurons with several antioxidants (Abe *et al.*, 1997; Henderson *et al.*, 1996; Ikeda *et al.*, 1995, 2000) delayed or improved—without totally correcting—the motor dysfunction. Excessive production of nitric oxide is another important risk factor for neurodegeneration of

FIG. 5. Low-power electron microscopy (4400×) of control and Wobbler mice ventral horn motor neurons. (A) Nucleus of a control motor neuron showing a nucleolus with its associated chromatin. The nuclear membrane shows an outer border surrounded by several mitochondria. (B) Degenerating motor neuron from untreated Wobbler mouse. Nuclear constituents are intact, without apoptotic fragmentation. However, massive perinuclear cytoplasmic vacuolation and irregular contour of the nuclear membrane characterize this cell. (C) Motor neuron from a progesterone-treated Wobbler mouse. Irregularity of the nuclear membrane is less pronounced than in the untreated Wobbler mice and perinuclear cytoplasmic vacuolation is markedly attenuated.



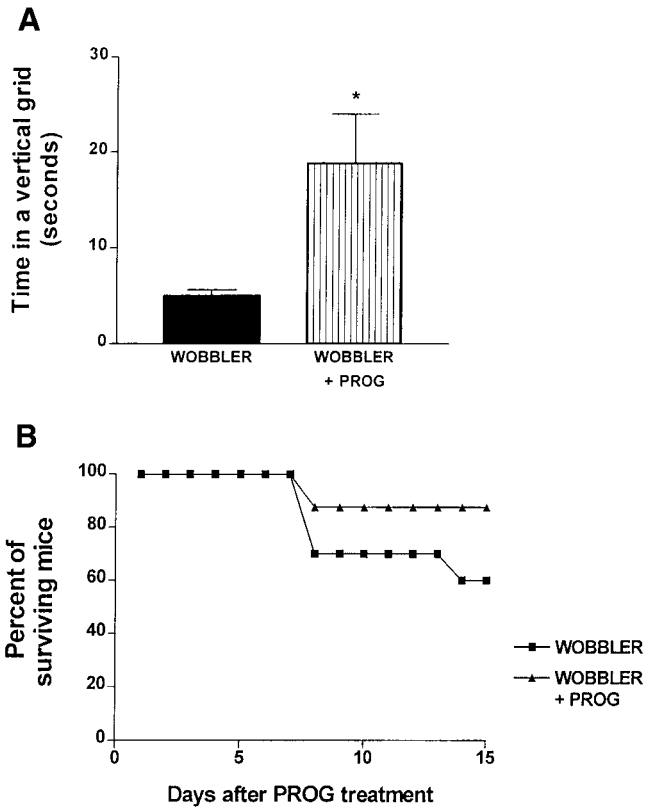


FIG. 7. Effect of progesterone treatment on muscle strength and survival time of Wobbler mice. (A) Time spent on a vertical grid was increased by progesterone treatment of Wobbler mice, in comparison to untreated mice ($n = 6$ animals per group, $^*P < 0.05$). (B) Survival time from day 0 (progesterone pellet implantation) to day 15 (time of sacrifice). On day 15, significantly more steroid-treated Wobbler mice survived compared to untreated mice ($n = 15$ animals per group; $P < 0.03$).

Wobbler mice, considering the increased activity of nitric oxide synthase (NOS) in ventral horn motor neurons (Clowry and McHanwell, 1996; González Deniselle *et al.*, 1999b) and the beneficial effects caused by NOS inhibition (Ikeda *et al.*, 1998). Therefore, NO toxicity may contribute to neurodegeneration, consider-

FIG. 6. High-power electron microscopy (20,000×) in control and Wobbler mice ventral horn motor neurons. (A) Control cell, showing several mitochondria with normal internal matrix, cristae, and outer membranes (arrows). (B) Degenerating motor neuron from an untreated Wobbler mouse, showing abnormal mitochondria with cristolysis and rupture of outer membrane (arrows). The asterisk marks a grossly abnormal, vacuolized mitochondria. The dark bodies in the lower edge of the photograph are phagosomes. (C) Motor neuron of Wobbler mouse receiving progesterone. The ultrastructure of some mitochondria is better conserved, including the cristae and outer membrane (arrows).

ing that after coupling with superoxide anion, NO forms peroxynitrites which damage lipids, proteins, and DNA.

Another important finding of the present experiments using symptomatic Wobbler mice was the pronounced reduction of the α3 subunit mRNA of the Na,K-ATPase in motor neurons. Increased lipid peroxidation was reported to dramatically reduce the enzyme activity (Hall and Braughler, 1982), but whether it also affects mRNA levels is unknown. Conceivably, this reduction would curtail pivotal functions of the α3 catalytic subunit of the enzyme such as Na/K exchange, maintenance of membrane potential, and neurotransmission (Lees, 1991; Stahl, 1986). Therefore, reduction of the Na,K-ATPase may exacerbate neuropathology, because the enzyme plays an important role for motor neuron function in the spinal cord. It should also be taken into account that the Na,K-ATPase may be one of several molecules changing in the spinal cord following PROG treatment, so recovery of this enzyme may be a partial view of a more complex situation. As the Wobbler disease essentially affected cholinergic motoneurons, an assay of the choline acetyltransferase (ChAT) may shed light favoring PROG neuroprotection. Preliminary assays of ChAT using the radiometric method of Fonnum (1975) demonstrated a 40% reduction in Wobbler mice cervical spinal cord, which increased toward normal after PROG treatment. New experiments are planned using immunocytochemistry and punch-out assays of the ventral horn to investigate the ChAT response to PROG in Wobbler mice of different age groups.

In this scenario, it was highly rewarding that a relatively short course of PROG treatment partially restored motor neuron morphology and also mRNA for the Na,K-ATPase α3 subunit in clinically affected Wobbler mice. At the molecular level, we suggest that at least three mechanisms might explain PROG neuroprotection. The first two were already discussed by Roof and co-workers (Roof and Hall, 2000; Roof *et al.*, 1994, 1997) to explain PROG beneficial effects against brain trauma and ischemia. One proposed mechanism is based on the antioxidant property of PROG, stabilizing plasma and intracellular membranes after intercalation between fatty acids components of phospholipids. This steroid ability could interfere with free radical attack on membranes (Hall and Braughler, 1982; Roof and Hall, 2000). Therefore, attenuation by PROG of oxygen-radical-induced lipid peroxidation may explain the decrease number of foamy cells, reduced vacuolation of individual cells, and partial recovery of mitochondrial morphology detected in Wobbler mice receiving PROG.

A second mechanism relies on the ability of PROG and its reduced derivatives to activate GABAa receptors, while inhibiting NMDA glutamate receptors (Rupprecht *et al.*, 1993). However, the actual importance of modulation of neurotransmitter receptors in PROG recovery of motor neuron morphology and function of Wobbler mice remains to be elucidated. In addition to the considered mechanisms, it is likely that PROG was acting through a third, genomic mechanism. Considering that a constitutive form of the PR is expressed by the spinal cord motor neurons (Labombarda *et al.*, 2000b), the possibility exists that PROG effects on morphological and biochemical parameters are transcriptionally mediated in the degenerating tissue.

In summary, restoration of motor neuron morphology and up-regulation of Na,K-ATPase subunit mRNA by PROG may afford neuroprotection in symptomatic Wobbler mice. The present work expands previous evidences for PROG protection using a spinal cord injury model (Labombarda *et al.*, 2001). However, it should be made clear that because the gene mutation in Wobbler mice is still unknown, it is impossible to ascertain whether neuroprotectants including steroids (Abe *et al.*, 1997; González Deniselle *et al.*, 1999b; Henderson *et al.*, 1996; Ikeda *et al.*, 1995; 1998; 2000; Tsuzaka *et al.*, 2001) target the elusive mutant gene or act on secondary consequences. Recently, Boillee *et al.* (2002) reported an early decreased level of the chaperone gene Msj-1 in the spinal cord and testis of Wobbler mice, suggesting its association with motoneuron degeneration. This finding may provide an approximation of the molecular action of different agents in the degenerative process.

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