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Regular Article

Progesterone restores retrograde labeling of cervical motoneurons in Wobbler mouse motoneuron disease

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Abstract

The Wobbler mouse, a mutant characterized by motoneuron degeneration in the cervical spinal cord, has been used to test the efficacy of novel treatments for human motoneuron diseases (HMD). Previous reports have shown that slow axonal transport is impaired in Wobblers and other models of HMD. Since progesterone (PROG) corrects some morphological, molecular, and functional abnormalities of Wobbler mice, we studied if steroid exposure for 8 weeks restored retrograde axonal transport by measuring motoneuron labeling after injection of fluorogold into the limb muscles. The dye was injected into forelimb biceps bracchii and flexor or into the rearlimb gastrocnemius muscles; 6 days later, the number of fluorescent motoneurons and the total number of cresyl violet stained motoneurons were counted in the cervical (C5–T1) or lumbar (L3–L5) spinal cord regions. A pronounced reduction (− 42.2%) of the percent of fluorescent motoneurons in Wobbler mice cervical cord was noted, which was significantly corrected after PROG treatment. In contrast, labeling of lumbar motoneurons was not reduced in Wobbler mice and was not affected by PROG treatment. In no case PROG showed an effect in control mice. Concomitantly, PROG slightly but significantly increased biceps weight of Wobbler mice. Behaviorally, PROG-treated Wobblers performed better on a motor test (hanging time from a horizontal rope) compared to untreated counterparts. We postulate a dual role for PROG in the Wobbler mouse, in part by prevention of motoneuron degeneration and also by enhancement of axonal transport. The latter mechanism could improve the traffic of neurotrophic factors from the forelimb muscles into the ailing motoneurons, improving neuromuscular function in this murine model of HMD.

Keywords: Axonal transport; Fluorogold; Motoneuron degeneration; Progesterone; Spinal cord; Wobbler mouse

Introduction

In the Wobbler mouse, a mutation mapped to chromosome 11 is responsible for motoneuron loss in the cervical spinal cord and brain stem (Duchen and Strick, 1968; Kaupmann et al., 1992; Mitsumoto and Bradley, 1982). The animal is considered a model for human motoneuron diseases (HMD) including amyotrophic lateral sclerosis (ALS) and infantile spinal muscular atrophy (Werdenig–Hoffman disease) (Cudkowicz and Brown, 1998; Junier et al., 1994; Price et al., 1994).

Phenotypic changes in Wobbler mice include progressive muscle weakness, tremor and ambulatory difficulty, muscle atrophy, and forelimb flexion (Duchen and Strick, 1968). Cervical motoneurons of Wobblers express genes involved in trophism and differentiation during embryonic life and exhibit vacuolation of cytoplasmic organelles, evidences of oxidative damage, increased nitrergic activity, and cytoskeletal disorganization with hyperexpression of the medium neurofilament (MNF) gene and retarded expression of neurofilament heavy (NFH) and light (NFL) chain mRNA

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(Junier et al., 1994; Junier et al., 1998; Popper et al., 1997; Pernas Alonso et al., 2001). Mitochondrial dysfunction, with decreases in complexes III and IV, is an early event in Wobbler mouse disease and is strongly implicated in motoneuron degeneration (Dave et al., 2003).

A pronounced axonal pathology is also typical of the disease. This includes impairment of slow axonal transport, decrease of neurofilament subunits and nerve terminals, secondary demyelination and diminished number and size of myelinated axons, presence of non-myelinating fibers of large diameter, and decrease in anterograde and retrograde axonal transport of proteins (Haenggeli and Kato, 2002; Mitsumoto and Gambetti, 1986). Using the fluorogold technique, Haenggeli and Kato (2002) have shown that, after dye injection into the biceps brachii and flexor muscles, the number of retrogradely labeled cervical motoneurons of Wobbler mice is reduced compared to wild-type animals. Impaired axonal transport also characterizes another model of ALS, the superoxide dismutase 1 (SOD 1) transgenic mouse, although in this case, the lumbar rather than the cervical motoneurons are primarily affected (Mohajeri et al., 1998).

A number of treatments, such as antiglutamatergic drugs, antioxidants, steroids, and neurotrophic factors show some degree of success to delay functional and/or biochemical abnormalities of Wobbler mice (Abe et al., 1997; González Deniselle et al., 1999; Ikeda et al., 1998; Ishiyama et al., 2004; Tsuzaka et al., 2001). In this respect, we have shown that progesterone (PROG), a steroid bringing neuroprotection to the injured peripheral (PNS) and central nervous system (CNS), is also effective in Wobbler mice (González Deniselle et al., 2002, 2004). Thus, neuropathology becomes less severe in Wobbler mice receiving PROG, with a reduction of vacuolated cells, preservation of mitochondrial ultrastructure, blockade of NO synthesis, regulation of gene expression, and enhancement of motor function and life span (González Deniselle et al., 2002, 2003, 2004). In the present report, we studied whether the beneficial effects of PROG in Wobbler mouse could be extended to axonal transport because of its close relationship to improvement of neuronal function. To this end, we employed the fluorogold dye method for labeling cervical and lumbar spinal cord motoneurons (Leong and Ling, 1990). The percent of fluorescent motoneurons from these areas was statistically compared between 4 experimental groups: controls, controls + PROG, Wobbler mice, and Wobbler mice + PROG. Additionally, we determined biceps weight in the four groups and compared muscle strength between PROG-treated Wobblers and steroid-naive animals.

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, USA, courtesy of Dr. Carl Hanson) and mated in our Institute. One-month-old homozygous Wobbler mice (*wr/wr*) were diagnosed as clinically affected Wobblers due to the presence of tremor, ambulatory difficulty, atrophy of forelimb digits, positive clasp knife reflex response, and diminished muscle strength, i.e. stage 1 of the disease according to Yung et al. (1992). Animals of both sexes were used in comparable numbers in all groups since neither the onset nor the progression of the disease correlated with sex. In the present study, there were no obvious differences between sexes in the effect of the treatment. At 6 weeks of age, a group of age-matched NFR control mice and Wobbler mice received under the skin of the neck a 20 mg PROG (Sigma) pellet under light ether anesthesia, whereas another group remained untreated. At 10 weeks of age, animals, which already received PROG, were reimplanted with a second steroid pellet. Thereafter, untreated and treated Wobbler mice, as well as similarly treated age-matched controls remained undisturbed. At the time of sacrifice, animals were 14 weeks old. 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.

Fluorogold uptake

Six days before sacrifice, the animals received injections of 2.5% fluorogold in distilled water (Fluorochrome Inc. CO, USA) via a 10 μl Hamilton syringe into either the rear or forelimb muscles. For labeling cervical motoneurons, one injection of 2.5 μl was made into the lower third part belly of the biceps bracchii muscle and 2 × 2.5 μl into one medial and one lateral flexor muscle of the right forearm after a midline incision to expose the muscles. For labeling lumbar motoneurons, a single tracer injection of 3 μl was made into the belly of the gastrocnemius muscle of the right rear limb. Different sets of animals were used for labeling cervical and lumbar motoneurons. Following injections, tracer leakage was removed from the surface of the muscles, the skin was sutured, and animals kept undisturbed until sacrifice.

The protocol of Haenggeli and Kato (2002) was followed to determine fluorogold uptake. Mice were heart perfused with 4% paraformaldehyde in phosphate-buffered 0.9% NaCl (PBS) and the spinal cords removed, post-fixed, and cryoprotected in 20% sucrose/PBS overnight. Thirty micrometer cryostat sections were mounted in glass slides and viewed without covers under the fluorescence microscope. Fluorogold labeled cells showing a bright fluorescent soma were identifiable as motoneurons based on location in Lamina IX of the ventral horn and size: >300 μm. This size corresponded to α motoneurons in the mouse (Mohajeri et al., 1998). Sections were examined under a Zeiss Axioplan Fluorescence Microscope, using BP 365 excitation, RKP 395 mirror, and LP 397 barrier filters, and retrogradely labeled motoneurons were counted and photographed in every

section at a 200× magnification. Photography was carried out with Kodak Ultra 400 film. Once fluorescent motoneurons were quantified, sections were stained with cresyl violet for counting the total number of motoneurons. A computerized image analysis program (Optimas Bioscan 6.02, Edmonton. WA, USA) was used for quantitative analysis of motoneurons. Results were expressed as % fluorescent motoneurons/total motoneurons localized in the ventral horn, Lamina IX, of the cervical or lumbar spinal cord. Approximately 60 sections were quantified for each cervical (C5–T1 level) and lumbar region (L3–L5) of the spinal cord. Each group was composed of 5–8 animals, and experiments were carried out at least 3 times. Data were statistically evaluated by one-way ANOVA followed by post-hoc Newman–Keuls multiple comparison test.

Progesterone levels in serum

Serum was obtained after blood collection from the heart at the time of sacrifice. The content of serum "progestins" in ng/ml was determined using a Coat-A-Count Progesterone kit (Diagnostic Products Corporation, Los Angeles, USA). PROG treatment produced a 5-fold increase in serum progestins: untreated mice: 7.9 ± 2.4 ng/ml; steroid-treated mice: 35.1 ± 3.7 ng/ml ($P < 0.001$). Thus, all steroid-treated mice were exposed to a high level of PROG during the 8-week period of study.

Behavioral performance

The effect of PROG treatment was studied in two ways. First, by determination of the biceps weight to the nearest milligram in a microgramatic balance and; second, by subjecting mice to a motor grip test to register motor performance (Bose et al., 1999). In this test, the animals were allowed to hold by the forelimbs from a horizontal rope tied to two vertical poles. The time in seconds spent by the animals hanging from the rope until they fell down was determined 3 times per animal and averaged. Animals were tested weekly for behavioral performance, and values obtained from 4 animals per group were pooled for statistical analysis. All data were reported as mean ± SE. In the grip test, a Student's t test was used to compare motor performance of untreated and PROG-treated Wobbler mice.

Results

As shown in Fig. 1, fluorescent motoneurons of the cervical region in control mice amounted to about 30% of total motoneurons; this value was not modified in controls + PROG. In contrast, Wobbler mice presented a significant 42% reduction in cervical motoneuron labeling (one way ANOVA, $F = 5.88$, $P < 0.01$ vs. controls). PROG treatment of Wobbler mice recovered most of the fluorogold labeling. In this group, although a small (10.4%) decrease of labeled

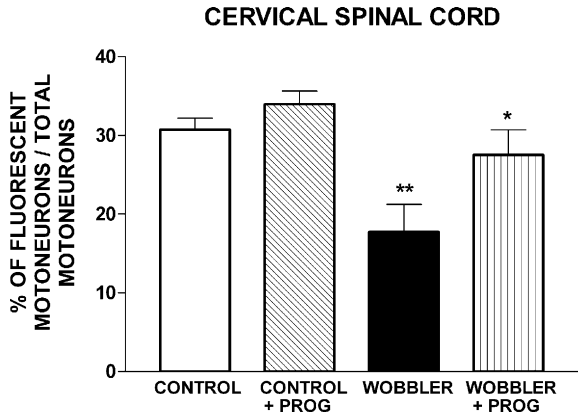

Fig. 1. Statistical comparison of the % fluorescent motoneurons/total motoneurons in the cervical region (C5–T1) of the spinal cord of control mice, control mice treated with progesterone (control + PROG) during 8 weeks, Wobbler mice, and Wobbler + PROG. To obtain these data, 2.5% fluorogold was injected into the biceps bracchii and flexor muscle, and mice were killed 6 days afterwards. Statistical analysis demonstrated a significant reduction of the percentage of labeled motoneurons in Wobblers with respect to control mice (**$P < 0.01$), while PROG treatment significantly increased % fluorogold labeled motoneurons in Wobbler (*$P < 0.05$ vs. Wobbler) but not in control mice (P: NS). Data were analyzed by ANOVA followed by the Newman–Keuls tests (n = 5–8 animals per group).

motoneurons was still present, it was not significantly different from the control or control + PROG groups. Instead, the PROG-treated Wobblers showed significantly more fluorescent motoneurons compared to the steroid-naive Wobblers ($P < 0.05$). Microscopic observations indicated that fluorescent motoneurons were positioned in clusters in the ventral horn, as shown in the representative photomicrographs of Fig. 2. This figure shows, from top to bottom, the fluorescent motoneurons in a control mouse (A), a control + PROG-treated mouse (B), the reduction in a Wobbler mouse (C), and the enhanced fluorogold labeling in a PROG-treated Wobbler mouse (D). Fluorescent cells of smaller size, probably corresponding to γ motoneurons (Mohajeri et al., 1998), were also observed but not quantified. Right panels of Fig. 2 correspond to the cresyl violet staining of the same animals shown on the left panel. This procedure was employed to calculate the % fluorescent cells in relation to the total number of motoneurons in the ventral horn and to characterize anatomically those cells taking up the dye by retrograde axonal transport.

Besides, an identical pattern was obtained if the number of fluorescent motoneurons for the 4 groups was analyzed per section, instead of referring to % of total motoneurons. Corresponding figures were: control: 5.7 ± 0.75 fluorescent cells/section, control + PROG: 5.7 ± 0.9, Wobbler: 2.9 ± 0.8 ($P < 0.001$ vs. control), and Wobbler + PROG: 4.3 ± 1.2 ($P < 0.05$ vs. untreated Wobbler, NS vs. control or control + PROG groups).

Consistent with the axonal transport studies, biceps muscle mass was reduced by 72% in untreated Wobbler mice (6.6 ± 0.7 g) compared to controls (23.8 ± 1.1 g). Although biceps atrophy was still present in Wobbler + PROG-treated mice, muscle

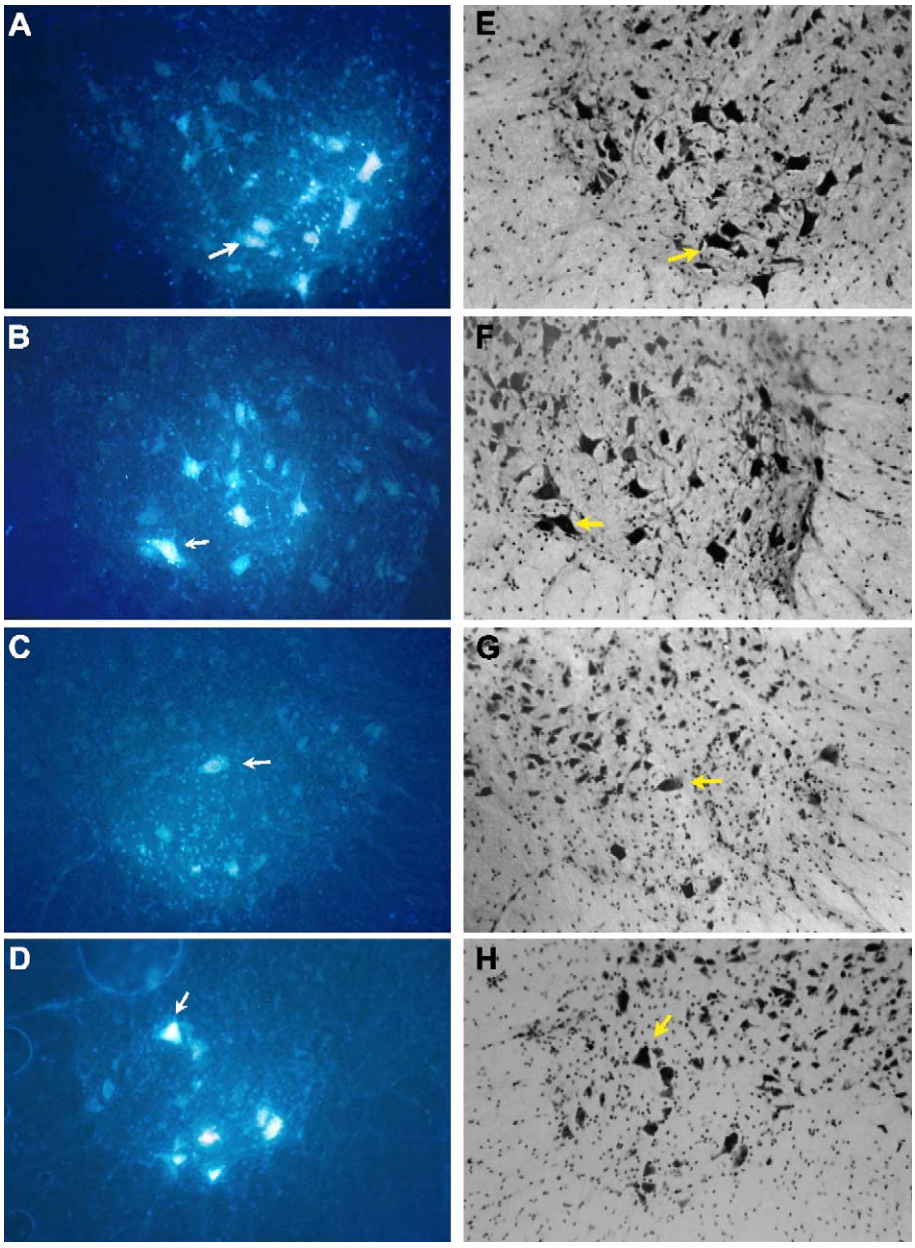

Fig. 2. Representative photomicrographs of labeled motoneurons in the cervical spinal cord after fluorogold injection into the biceps bracchii and flexor muscle of the forelimbs. Note the cluster of large fluorescent motoneurons in the ventral horn in a control (A) and control + PROG-treated mouse (B), a noticeable decrease in labeled motoneuron neurons in an untreated Wobbler mouse (C), and the restoration produced by PROG treatment in a Wobbler mouse (D). (E–H) Corresponding cresyl violet staining of ventral horns of the animals depicted in photographs (A–D). In corresponding photomicrographs (i.e. A–B, etc), arrows identify the same neurons. Magnification: 200×.

weight was significantly increased with respect to steroid naive Wobblers (11.1 ± 1.3 g, $P < 0.05$). Behaviorally, as a test of muscle function, we carried out experiments to compare the hanging time from a horizontal rope between steroid-naive and PROG-treated Wobbler mice. Fig. 3 shows that untreated Wobblers almost immediately dropped from the rope, while their PROG-treated counterparts spent an average hanging time of ≈4 s without overlapping between the two groups. The experiment was repeated three times with similar results.

In contrast to findings in the cervical region, however, quantitation of the number of fluorescent lumbar motoneurons after injection of flurorogold into the gastrocnemius muscle did not show significant differences among the 4 groups of mice (Fig. 4).

Discussion

The results of this investigation agree with previous reports showing decreased retrograde axonal transport and impairment of fluorogold uptake into the cervical but not lumbar spinal cord motoneurons of Wobbler mice (Mitsumoto and Gambetti,

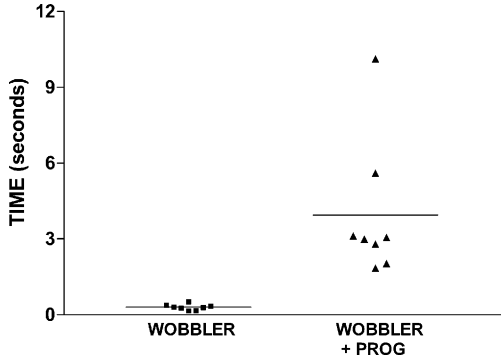

Fig. 3. Behavioral test to determine grip strength in Wobbler mice with and without PROG treatment. Animals were placed on a rope tied to two vertical poles, and the hanging time was recorded. Mice were tested weekly for behavioral performance. Untreated Wobbler mice were unable to hang from the rope for longer than 0.30 ± 0.04 s (mean ± SE), whereas PROG-treated mice hang for at least 4.0 ± 0.96 s ($P = 0.002$ by Student's t test). No group overlapping was noticeable.

1986; Haenggeli and Kato, 2002). Importantly, we generated data regarding a new effect of PROG. Thus, PROG treatment significantly enhanced fluorogold uptake of spinal cord cervical motoneurons in Wobbler mice but not in control mice. The lack of steroid effect in controls was not unexpected, in agreement with previous results. Using the spinal cords of NFR control mice, we have been unable to observe a PROG effect on parameters otherwise regulated by PROG in the Wobbler mouse (NADPH-diaphorase/NOS activity and the Na,K-ATPase mRNA) (González Deniselle et al., 2002, 2003, 2004). In a spinal cord injury model, PROG enhancement of choline acetyl-transferase, Na,K-ATPase mRNA, GAP-43 mRNA, and BDNF mRNA and protein occurred in injured but not control rat spinal cord (Labombarda et al., 2002; González et al., 2004). Therefore, it is likely that injury and neurodegeneration created a permissive environment for PROG effects, including the axonal retrograde transport.

To interpret the mechanisms of PROG effects on axonal transport, it seems important to recall the anatomical pathway of fluorogold uptake. After dye injection into muscles, certain reactive groups of the tracer facilitate endocytotic vesicular uptake at the nerve terminals (Schmued and Fallon, 1986). From the terminals, fluorogold is retrogradely transported via slow axonal transport to reach the parent motoneuron (Leong and Ling, 1990). It follows that PROG effects could take place at the muscle, the nerve terminal, the axon, or the motoneuron. In addition, since the tracer was injected into the muscles, these results could imply a more functional neuromuscular junction in Wobblers receiving PROG than untreated Wobblers (Haenggeli and Kato, 2002). Some authors consider motoneurons the primary targets of the Wobbler mutation, whereas axon pathology, demyelination, and muscle atrophy are viewed as secondary events (Boillée et al., 2003). In this respect, different findings support that the Wobbler disease is a neuronopathy: a—cell soma degeneration precedes that of the axons and b—the occurrence of early mitochondrial pathology in motoneurons (Boillée et al., 2003; Dave et al., 2003). Therefore, the decreased fluorogold labeling in clinically afflicted Wobbler mice might reflect the extent of motoneuron degeneration, whereas the gain in the number of fluorescent cells in the Wobbler + PROG group might indicate enhanced motoneuron function/survival. PROG treatment of Wobbler mice during early stages of the disease corrects molecular and biochemical abnormalities of motoneurons, reduces mitochondrial pathology and the generation of levels of NO toxic to the respiratory chain (González Deniselle et al., 2002, 2004). Thus, effects of PROG possibly take place directly upon motoneurons.

Other reports, in contrast, emphasized that the impairment of axonal transport in Wobbler mice is independent of the concomitant neuronopathy. For example, impaired axonal transport is much more widespread than the neuronal abnormalities (Mitsumoto and Gambetti, 1986), whereas accumulation and abnormal expression of neurofilaments in axons could trigger the neuronal degeneration (Pernas Alonso et al., 2001). Therefore, PROG action might also involve the axonal projections. The possibility for axonal effects is supported by reports of PROG neuroprotection in the peripheral nervous system. Thus, in peripheral nerves following injury and in the degenerating nerves of old animals, PROG exerts a stimulatory effect on gene expression of the myelin proteins P0 and PMP22, increases axonal diameter, and impulses regeneration (Azcoitia et al., 2003; Desarnaud et al., 1998; Melcangi et al., 2000). Whatever the favored mechanism, PROG treatment of Wobbler mice resulted in a slight increase in biceps weight and increased hanging time, a measure of enhanced muscle strength.

Therefore, PROG treatment could play a dual role in Wobbler mice. On one side, preserving motoneurons from degeneration and death by paraptosis (Clarke, 1990); on the

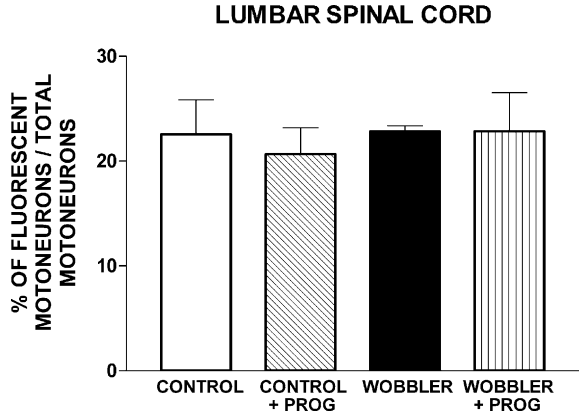

Fig. 4. Statistical comparison of the % fluorescent motoneurons/total motoneurons in the lumbar region (L3–L5) of the spinal cord of control mice, control mice treated with progesterone (control + PROG) during 8 weeks, Wobbler mice, and Wobbler + PROG. Data were obtained 6 days after injection of 2.5% fluorogold into the gastrocnemius muscle. In contrast to differences found in the cervical region, there were no significant differences between the 4 groups in motoneuron labeling in the lumbar spinal cord.

other, enhancing axonal transport from the forelimb muscles. From the therapeutic point of view, the latter effect could be useful to increase the transfer of neurotrophins and other factors from the periphery into the ailing motoneurons of murine models and patients with HMD. Advantage may be taken of this peculiar effect of PROG to test if, combined with other therapies (Abe et al., 1997; Ikeda et al., 1998; Ishiyama et al., 2004; Tsuzaka et al., 2001), it substantially avoids the progression of motoneuron dysfunction in the Wobbler mouse.

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