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ORIGINAL ARTICLE

Progesterone prevents mitochondrial dysfunction in the spinal cord of wobbler mice

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Abstract

In the Wobbler mouse, a mutation of the Vps54 protein increases oxidative stress in spinal motoneurons, associated to toxic levels of nitric oxide and hyperactivity of nitric oxide synthase (NOS). Progesterone neuroprotection has been reported for several CNS diseases, including the Wobbler mouse neurodegeneration. In the present study, we analyzed progesterone effects on mitochondrial-associated parameters of symptomatic Wobbler mice. The activities of mitochondrial respiratory chain complexes I, II-III and IV and protein levels of mitochondrial and cytosolic NOS were determined in cervical and lumbar cords from control, Wobbler and Wobbler mice receiving a progesterone implant for 18 days. We found a significant reduction of complex I and II-III activities in mitochondria and increased protein levels of mitochondrial, but not cytosolic nNOS, in the cervical cord of Wobbler mice. Progesterone treatment prevented the reduction of complex I in the cervical region and the increased level of mitochondrial nNOS. Wobbler motoneurons also showed accumulation of amyloid precursor protein immunoreactivity and decreased activity and immunostaining of MnSOD. Progesterone treatment avoided these abnormalities. Therefore, administration of progesterone to clinically afflicted Wobblers (i) prevented the abnormal increase of mitochondrial nNOS and normalized respiratory complex I; (ii) decreased amyloid precursor protein accumulation, a sign of axonal degeneration, and (iii) increased superoxide dismutation. Thus, progesterone neuroprotection decreases mitochondriopathy of Wobbler mouse cervical spinal cord.

Keywords: mitochondria, motoneuron degeneration, neuroprotection, progesterone, Wobbler mouse.

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The Wobbler mouse is a model for the sporadic form of amyotrophic lateral sclerosis (ALS). Abnormalities of Wobbler mouse spinal cord include motoneuron degeneration, astrogliosis and activation of microglia (Mitsumoto and Bradley 1982; Hantaz-Ambroise *et al.* 1994; Boillée *et al.* 2003). Genetically, Wobblers bear a mutation in the gene coding for VPS_{54} (vacuolar vesicular protein sorting) localized in chromosome 11 (Rathke-Hartlieb *et al.* 1999). In the past, Wobblers have been extensively employed in pharmacological trials intended to alleviate neurodegeneration of humans with ALS and spinal muscular atrophy. Thus, clinical, biochemical and morphological improvement have been reported after treatment of Wobblers with antioxidants, antiglutamatergic drugs, neurotrophic factors, steroids and nitric oxide inhibitors (Henderson *et al.* 1996; Ikeda *et al.* 1998, 2000; Gonzalez Deniselle *et al.* 2001; Tsuzaka *et al.* 2001).

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Abbreviations used: ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; BSA, bovine serum albumin; ISH, *in situ* hybridization; MnSOD, manganese Superoxide Dismutase; mtNOS, mitochondrial nitric oxide synthase; nNos, neuronal nitric oxide synthase; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; PFA, paraformaldehyde; SSC, sodium citrate/sodium chloride buffer.

Recently, the usefulness of Wobbler mice as a model for ALS has been re-evaluated, due to findings common to rodents and humans that include abnormalities in the expression and localization of TDP43 (transactive response DNA-binding protein), axonal accumulation of amyloid precursor protein (APP), changes of ubiquitination, elevated expression of the transcription factor p53, muscle proteomics, cortical hyperexcitabilty and clinical effectiveness of Riluzole (Fumagalli *et al.* 2006; Eve *et al.* 2007; Dennis and Citron 2009; Nieto-Gonzalez *et al.* 2011; Palmisano *et al.* 2011; Staunton *et al.* 2011).

Motoneuron abnormalities of Wobbler mice have been the subject of several studies. Morphologically, anterior horn motoneurons of the cervical region of the spinal cord undergo a dramatic perikaryal vacuolar degeneration (Mitsumoto and Bradley 1982), accompanied by astrocytosis (Laage *et al.* 1988; Gonzalez Deniselle *et al.* 2001; Hantaz-Ambroise *et al.* 1994) and microglial activation (Boillée *et al.* 2003). These neuropathological changes resemble the type II or cytoplasmic form of cell death (Clarke 1990). Participation of oxidative stress in this mechanism is supported by abnormalities of mitochondrial function in Wobbler mice. Under pathological conditions, mitochondria are a source of free radicals, superoxide anion and nitric oxide due to the high activity of a mitochondrial nitric oxide synthase (mtNOS) (Finocchietto *et al.* 2009). Of critical importance is the production of toxic levels of NO in the Wobbler mouse (Clowry and McHanwell 1996). Excess levels of NO bind to and alter the function of some components of the respiratory chain (Carreras *et al.* 2004). In mitochondria from the spinal cord and in some cases motor cortex of Wobbler mice, there are demonstrations of respiratory chain dysfunction with decreased oxygen consumption, decreased complex I and complex IV activities, decrease in states 3 and 4 respiration rates, decreased oxidative phosphorylation and aberrant activation of delta Protein kinase C that modulates mitochondrial-induced apoptosis (Xu *et al.* 2001; Dave *et al.* 2003a,b; Santoro *et al.* 2004). At the ultrastructural level, vacuolated motoneurons from Wobbler mice present mitochondrial membrane disruption, cristolysis and vacuolation (Gonzalez Deniselle *et al.* 2002). Considering the significant role played by mitochondria in cell metabolism and function, it seems important to clarify which factors could prevent the mitochondriopathy of the Wobbler mouse spinal cord.

Our laboratory has reported the neuroprotective effects of progesterone using models of spinal cord injury, Wobbler mouse neurodegeneration, neuroinflammation and neurophatic pain (reviewed in De Nicola *et al.* 2009). Progesterone neuroprotective effects have been also demonstrated in traumatic brain injury, stroke, ischemia and in peripheral neuropathy of traumatic or diabetic origin (Melcangi and Panzica 2009; Sayeed and Stein 2009; Wang *et al.* 2010; Stein 2011). Progesterone treatment of Wobbler mice has shown neuroprotective and astroglial inhibitory responses, such as decreased number of vacuolated motoneurons, reduction in the density of NOS-active neurons and astrocytes, enhanced morphology of mitochondria, increased expression of brain derived neurotrophic factor in motoneurons and oligodendrocytes, restoration of cholinergic neurotransmission and of axonal transport (Gonzalez Deniselle *et al.* 2002, 2004, 2007; Meyer *et al.* 2012). Clinically, chronic progesterone administration attenuates the atrophy of Wobbler mice forelimb biceps bracchii, increases muscle strength and prolongs survival.

The aim of the present studies was to elucidate if progesterone normalized the aberrant mitochondrial function of Wobbler mice. This possibility received support from reports showing the protective effects of progesterone for mitochondrial function. Thus, progesterone and its metabolite allopregnanolone inhibit the mitochondrial permeability transition pore, which opening allows the release of cytochrome C into the cytosol and the initiation of apoptotic events (Sayeed *et al.* 2009). Additionally, progesterone reduces lipid peroxidation, decreases the rate of reactive oxygen leak and increases respiratory activity of brain mitochondria from ovariectomized rats (Irwin *et al.* 2008) and reverses in low doses the mitochondrial dysfunction in rats with brain trauma (Robertson *et al.* 1996).

In the present report, we first determined the activities of complex I, II-III and IV in mitochondria isolated from the cervical and lumbar spinal cords of control and Wobbler mice with and without progesterone treatment. Second, using western blot, we compared levels of neuronal nitric oxide synthase (nNOS) in mitochondria and cytosol isolated from the cervical and lumbar spinal cords from control and Wobbler mice with and without progesterone treatment. Third, using *in situ* hybridization (ISH) and real time PCR (qPCR), the expression of nNOS mRNA was measured in the spinal cord from the groups mentioned above. Fourth, we determined the activity and immunostaining of the manganese superoxide dismutase (MnSOD), considering the antioxidant function of this enzyme. Finally, we studied if a block in anterograde axonal transport could explain nNOS retention in the cytoplasm, hypothetically explaining its mitochondrial transfer. To accomplish this objective, the number of APP immunostained axons – an index of impaired axonal transport – was counted in control, steroid-naive and progesterone-treated Wobbler mice. The results confirm an important role of progesterone to arrest the mitochodriopathy of Wobbler mice spinal cord.

Materials and methods

Materials

Ensure was obtained from Abbott Argentina. Purogene Core Kit A was used for mouse genotyping (Qiagen Sciences, Germantown, MD, USA). Progesterone, NADH, NADPH, cytochrome c, succi-

nate, l-malic acid, nitroblue-tetrazolium and rotenone were obtained from Sigma (St Louis, MO, USA). Oligonucleotide probes were synthesized by Oligos ETC. Inc. (Wilsonville, OR, USA). The enzyme terminal transferase was purchased from Boehringer-Mannheim (Germany). Dektol, Ektaflo fixer and NTB 2 autoradiography emulsion were products of Kodak (New York, NY, USA). Trizol, Dnase I and SuperScript III Rnase H reverse transcriptase kit were obtained from InvitroGen Life Technologies (Carlsbad, CA, USA). Sources of commercial antibodies were as follows: manganese Superoxide Dismutase (MnSOD) polyclonal antibody from Stressgen (cat. no. SOD-110; Enzo Life Sciences, Farmingdale, NY, USA), anti-APP monoclonal antibody Clone 22C11 from Chemicon Millipore (Billerica, MA, USA) and polyclonal rabbit nNOS N30130 from Becton Dickinson (Franklin Lakes, NJ, USA). The ABC kit for immunocytochemistry was obtained from Vector Labs (Burlingame, CA, USA). All other chemicals used in this investigation were reagent grade.

Experimental animals and treatments

Control (NFR/NFR) and Wobbler mice (wr/wr) obtained from the Instituto de Biología y Medicina Experimental colony were kept under conditions of controlled humidity and temperature (22°C), with lights on from 07:00 AM to 07.00 PM and fed standard mice chow supplemented with a protein, mineral and vitamin nutrient mixture (Ensure). The Wobbler phenotype was identified according to Rathke-Hartlieb *et al.* (1999), employing an Alu I restriction polymorphism of a Cct4 amplification product for testing the allelic status at the wr locus. Genotyping demonstrated a 20% incidence of the wr/wr genotype in different litters. In contrast to patients with ALS that show a gender difference in disease incidence and clinical features (McCombe and Henderson 2010), we have not found gender differences in Wobbler mice. Accordingly, animals of both sexes were used in comparable numbers in all groups, because neither the onset nor the progression of the disease correlated with sex (Gonzalez Deniselle *et al.* 2005).

Animals were housed in group cages containing 2–3 Wobblers and one control mouse. This social interaction plus supplementing the special nutrient prolonged the life span and improved the health status of the Wobbler mice (M-P. Junier, personal communication) Wobbler mice at the initial symptomatic stage (2–3 months old) remained untreated or received s.c. a single progesterone pellet (20 mg), prepared as previously described (Meyer *et al.* 2010) for exactly 18 days. Animal procedures followed the Guide for the Care and Use of Laboratory Animals (NIH Guide, Instituto de Biología y Medicina Experimental Assurance Certificate No. A5072-01) and were approved by the Institute's Animal Care and Use Committee. Efforts were made to keep the number of animals at a minimum and to diminish animal discomfort.

Determination of vacuolated motoneurons

Motoneuron vacuolation density was measured according to Gonzalez Deniselle *et al.* (2004). Following deep anesthesia with a mixture of xylazine (6 mg/kg) and ketamine (75 mg/kg), mice were perfused transcardially with a solution containing 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer pH 7.4. Following laminectomy, cervical spinal cords were removed and small blocks of tissue were obtained by cutting transverse sections of 2–3 mm maximum length. Blocks were immersed for 2.5 h in 4% PFA, post-fixed in graded ethanol and embedded in paraffin. Paraffin sections (5 μm) were stained with cresyl violet for light microscopy observations and quantitation of cells showing cytoplasmic vacuolation. Sections were photographed using an Axiophot Zeiss light microscope. The number of vacuolated motoneurons was quantified in eight sections per animal ($n = 7$ animals per group) in Lamina IX ventral horn using a computerized image analysis system (Bioscan Optimas 6.02). Results were expressed as the number of vacuolated motoneurons/mm^2 of ventral horn area (mean ± SEM).

Subcellular fractionation

The cervical and lumbar regions of the spinal cord were homogenized in 320 mM sucrose/20 mM HEPES, pH 7.2, containing 1 mM EDTA, 1 mM dithiothreitol, 10 μg/mL leupeptin, 2 μg/mL aprotinin, and 10 μg/mL phenylmethylsulfonyl fluoride. Mitochondria was purified by Percoll gradient centrifugation (Giulivi *et al.* 1998) in a buffer consisting of 0.23 M mannitol, 0.07 M sucrose, 0.5 mM EGTA, and 2 mM HEPES, pH 7.4. The cytosolic fraction was obtained after 100 000 *g* centrifugation of the 8000 *g* supernatant of spinal cord homogenates.

Western blotting

Aliquots containing 100 μg of cytosolic or mitochondrial protein were separated by electrophoresis on reducing 6% sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were washed with 0.1% Tween 20 in 20 mM Tris buffer, 137 mM NaCl, pH 7.4, and blocked with 5% non-fat milk. Membranes were incubated with a nNOS rabbit purified antibody for 1 h at 25°C or overnight 4°C at a 1 : 1000 dilution, washed, and subsequently incubated with a secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase (1 h, 1 : 3000 dilution). Bands were detected by chemiluminescence using an enhanced chemiluminescence kit and quantified by measuring the optical density. Quantification of bands was performed by digital image analysis using a Hewlett-Packard scanner and Totallab analyzer or Image J software (Nonlinear Dynamics, Biodynamics). Results were derived from $n = 3$ separate experiments, each one comprising a pool of 12 animals per group.

Measurement of mitochondrial complex activities

The activity of complex I was followed by the rotenone-sensitive reduction of NADH absorbance at 340 nm that leads to the reduction of ubiquinone to ubiquinol. The activity is expressed as nmoles/ min.mg protein. The activity of complex II-III was measured by the increase in absorbance at 550 nm due to the reduction of cytochrome *c* in the presence of succinate. The activity is expressed in nmoles/min.mg prot. Complex IV activity was followed by the oxidation of cytochrome c measured as a decrease in absorbance at 550 nm, and measured as the pseudo first order constant *k/min.mg prot* or as nmoles oxidized cyt c/min.mg protein (Cardoso *et al*. 1999; Wharton and Tzagoloff 1967). Mitochondrial complex activities were expressed as % deviation (mean ± SEM) from control. Results were derived from $n = 3$ separate experiments, each one comprising a pool of 12 animals per group.

In situ hybridization for nNOS mRNA

Spinal cords were immediately removed following dorsal laminectomy, post-fixed for 2 h at 4°C in 4% PFA, cryoprotected by immersion

in 20% sucrose overnight and kept frozen at −80° C until used. *In situ* hybridization was carried out under Rnase-free conditions following published protocols (Gonzalez Deniselle *et al.* 2002). Cryostat sections obtained at the cervical level of the spinal cord were fixed in 2% PFA, washed in 0.5× sodium citrate/sodium chloride buffer (SCC; 1× SCC: 0.15 M sodium chloride/ 0.015 M sodium citrate, pH 7.0), dried and acetylated with acetic anhydride. To detect nNOS mRNA, a 45 mer synthetic oligonucleotide probe with the sequence 5′GCCTTGGGCATGCTGAGGGCCATTACCCAGACCTGTGA CTCTGTC3′ was end-labeled with (^{35}S)dATP using the enzyme terminal transferase. Hybridization was carried out using 10^6 cpm of ^{35}S-labelled probe in 100 µL hybridization cocktail containing: 0.02% Ficoll 400, 0.02% polyvinylpirrolidone, 0.02% bovine serum albumin (BSA), 50% formamide, 3× SCC 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 sulphate. After overnight hybridization at 42°C, sections were washed several times in SCC, dried, and dipped into Kodak NTB-2 emulsion and exposed in the dark for 45 days. Sections were then developed with Dektol (1 : 2 dilutions with water), fixed in Ektaflo fixer, counter-stained with cresyl violet and coverslipped with Permount. For the semi-quantitative evaluation of nNOS mRNA, the number of silver grains per cell was determined over motoneurons of ventral horn Lamina IX measuring 400–600 µm^2 and showing a clear nuclear profile. Grain counting was performed in an Olympus microscope coupled to an image analysis system (Bioscan Optimas 6.02) and calculated after background subtraction. Results were expressed as the mean grain density (number of grains per unit area of soma: grains/µm^2) ± SEM. Data from four sections per mice and at least 12 motoneurons per section were combined to obtain a mean value per animal. The number of animals was five per group.

Real-time PCR of nNOS

For real-time PCR, a 0.5-cm segment of the cervical spinal cord was removed and homogenized with a Polyitron homogenizer. Total RNA was then extracted using Trizol reagent. The concentration and purity of total RNA was determined by measuring the optical density at 260 and 280 nm. All samples were precipitated with ethanol and then dissolved in distilled water at a concentration of 1 µg/µL. Total RNA was subjected to Dnase 1 treatment (2 U for 10 min at 25°C) to remove residual contaminating genomic DNA. cDNA templates for PCR amplification were synthesized from 2 µg of total RNA using a SuperScript III Rnase H reverse transcriptase kit for 60 min at 42°C in the presence of random hexamer primers. Primer sequences for nNOS were: foward 5′-ACCAGCC ATTAGCAGTAGAC-3′; nNOS reverse 5′-AGCAGTCATCCT GTTCTCC-3′. Cyclofilin B was used as a housekeeping gene. The relative gene expression of nNOS mRNA was determined using the ABI PRISM 7500 sequence Detection System (Applied Biosystems, Foster City, CA, USA). Relative gene expression data were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), and it was determined as fold induction with respect to its respective control. For each amplification, 2 ng cDNA/µL of reaction was used and PCR was performed in triplicate under optimized conditions: 95°C at 10 min followed by 40 cycles at 95°C for 0.15 s and 60°C for 1 min. Primer concentration was 0.4 µM.

Results were expressed as mean fold increase ± SEM of six animals per group.

NADPH-diaphorase histochemistry

This activity, based on the diaphorase property of NOS, was determined by the procedure of Vincent and Kimura (1992) in 16 µm cryostat sections of the cervical spinal cord. The reaction is based on the reduction of nitroblue-tetrazolium to its insoluble formazan reaction product with purple-black staining of the site enzyme loication. Slices 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 containing: 0.2 mg/mL of nitroblue tetrazolium, 2.7 mg/mL L-malic acid, 1 mg/mL of β-NADPH, 0.3% Triton X-100 dissolved in 0.1 M Tris–HCl buffer (pH 7.4). After keeping the reaction in the darkness during 60 min at 37°C, it was stopped by two washes in PBS at 25°C. Sections were then dehydrated briefly in ethanol, air dried and coverslipped with Permount.

Detection of MnSOD enzyme activity and expression by immunofluorescence

MnSOD was determined by inhibition of the rate of 20 µM cytochrome *c* reduction by 0.5 mM xanthine and xanthine oxidase (initial rate: 0.025 A/min), as reported by Boczkowski *et al.* (1999). The assay was carried out in 50 mM potassium phosphate and 0.1 mM EDTA (pH 7.8) at 550 nm. Sodium cyanide (1 mM) was used to inhibit Cu-Zn-SOD and cytochrome oxidase activities. Three separate experiments were carried out to determine MnSOD activity, each one comprising a pool of 12 animals per group.

For MnSOD immunoflourescence, 30 µm cryostat sections were incubated with 3% goat serum during 10 min at 37°C, and followed by overnight incubation at 4°C with a 1/250 dilution of the MnSOD polyclonal antibody prepared in 2% goat serum, 0.5% Triton-X100 in PBS. After washing, sections were incubated with a 1/1000 dilution of a goat-anti rabbit IgG Alexa 488 in 2% goat serum, 0.5% Triton X-100 during 1 h at 25°C. Sections were washed, mounted with Fluoromont and examined under a Nikon Eclipse E 800 confocal scanning laser microscope equipped with Nikon 11691 photographic equipment. Photographs were taken at 600× magnification. Results were obtained from 10 sections per animal, $n = 7$ animals per group (mean ± SEM).

APP immunocytochemistry

APP protein immunstaining was determined according to Linker *et al.* (2005). Mice were transcardially perfused with 4% PFA and cervical spinal cords embedded in paraffin. Five-micrometreslices were first incubated with 3% H_20_2 in methanol to block endogenous peroxidase. For antigen retrieval, slices were microwaved for 5″ and then pre-incubated in 10% BSA. For immunocytochemistry, we employed a 1/1000 dilution of an anti-APP monoclonal antibody in PBS containing 10% BSA during 20 h at 4°C. As second antibody, we used a biotin-conjugated goat antimouse IgG (diluted 1/200 in 1% BSA). Slices were further processed according to the ABC kit instructions and developed with 3,3′-diaminobenzidine 0.5 mg/mL, 0.05% H_2O_2. Sections were counter-stained with cresyl violet, dehydrated, cleared and mounted with Permount Non-specific staining was assessed in the absence of primary antibody. The number of APP-positive neurons was determined in ventral horn using a computarized image analysis system (Bioscan, Optimas 6.02). Ten sections of the spinal cord per animal were analyzed to determine the number of neurons per unit area containing APP

immunoreactivity in the cell bodies ($n = 10$ animals per experimental group). Data were expressed as number of APP + profiles per section (mean ± SEM).

Statistical analysis

All results were expressed as mean ± SEM. Data were analyzed by one-way ANOVA followed by the *post hoc* Newman–Keuls test when three groups were compared or two-tailed Student's *t*-test if only two groups were analyzed. Statistical analyses were performed with Prism 4 GraphPad software (San Diego, CA, USA). Significance was set at $p < 0.05$.

Results

Progesterone reduces vacuolated motoneurons of Wobbler mice

In agreement with previous observations, several vacuolated motoneurons were present in the spinal cord ventral horn from Wobbler mice, whereas none were present in the control NFR/NFR animals (Fig. 1b). Progesterone treatment significantly decreased vacuolated motoneurons (Wobblers: 26.26 ± 1.77; Wobblers + progesterone: 19.20 ± 1.98/unit area; $p < 0.05$, Student's *t*-test) (Fig. 1a). As vacuolated motoneurons originate from intense oxidative stress, this finding clearly indicates that progesterone opposed oxidative damage to intracellular membranes and organelles.

Progesterone decreases the high expression of mtNOS from Wobbler mouse

NO is a likely candidate responsible for vacuolation of motoneurons, because as a free radical, it damages mitochondria with further generation of free radicals and oxidative damage. Therefore, we compared by western blot the expression of nNOS in isolated mitochondria and cytosol from the cervical and lumbar regions of the spinal cord of control and Wobbler mice ± progesterone treatment. Figure 2a shows an increased signal for nNOS in mitochondria (mtNOS) from the cervical, but not lumbar spinal cord, of steroid-naïve Wobblers compared with control and progesterone-receiving Wobblers. In contrast, cytosolic nNOS in the same groups seemed unchanged in the cervical portion and weakly present in the lumbar region (Fig. 2b). Quantitative densitometric analysis confirmed higher content of mtNOS in cervical spinal cord mitochondria from Wobbler mice ($p < 0.05$), which was normalized by progesterone treatment (Fig. 2c). In the lumbar region, untreated Wobblers showed a small, non-significant reduction of mtNOS, which was unmodified by progesterone. Cytosolic nNOS content from the cervical or lumbar spinal cord was similar under the different experimental conditions (Fig. 2d). Immunocytochemistry for NADPH-diaphorase (NOS) demonstrated several staining profiles in the untreated Wobbler, which may correspond to motoneurons and endothelial cells (arrows, Fig. 2e). Staining of motoneurons was absent in the control and progesterone-treated Wobbler.

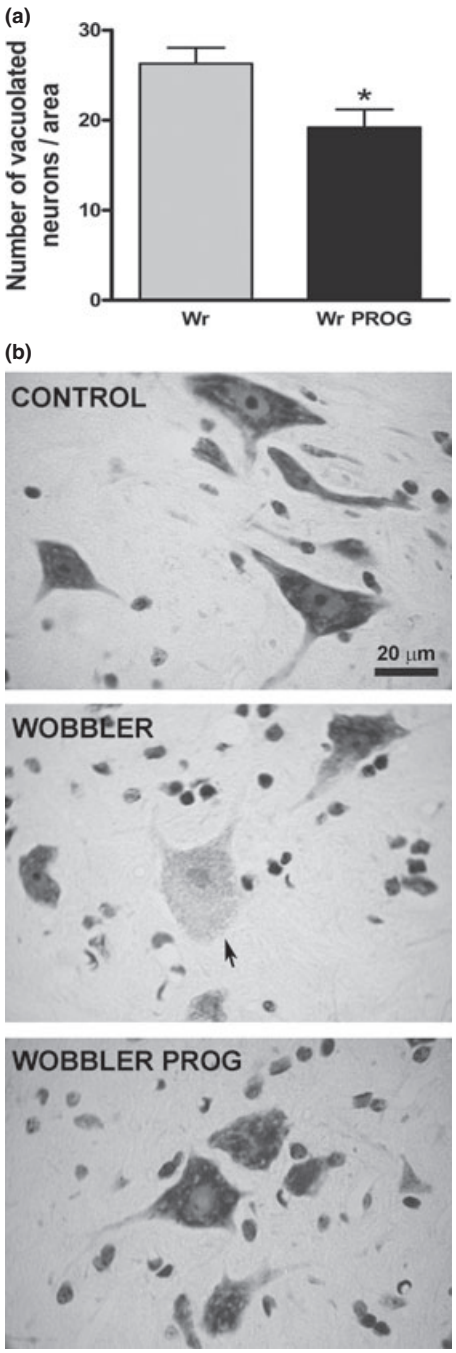

Fig. 1 (a) Number of vacuolated motoneurons per unit area (mm^2) in untreated Wobbler mice (gray column) and Wobbler mice receiving progesterone during 18 days (black column). Progesterone treatment significantly decreased motoneuron vacuolation (*$p < 0.05$, Student's *t*-test). Figures represent the average counting of eight sections per animal, $n = 7$ animals per group. (b) Absence of vacuolated motoneurons in a control mouse spinal cord (upper image). Vacuolated motoneurons characterized the ventral horn from an untreated Wobbler mouse (arrow, middle image). The lower image shows a reduction of motoneuron vacuolation in a progesterone-treated Wobbler. Microphotographs were taken from the cervical region of the spinal cord. Inside bar: 20 µm.

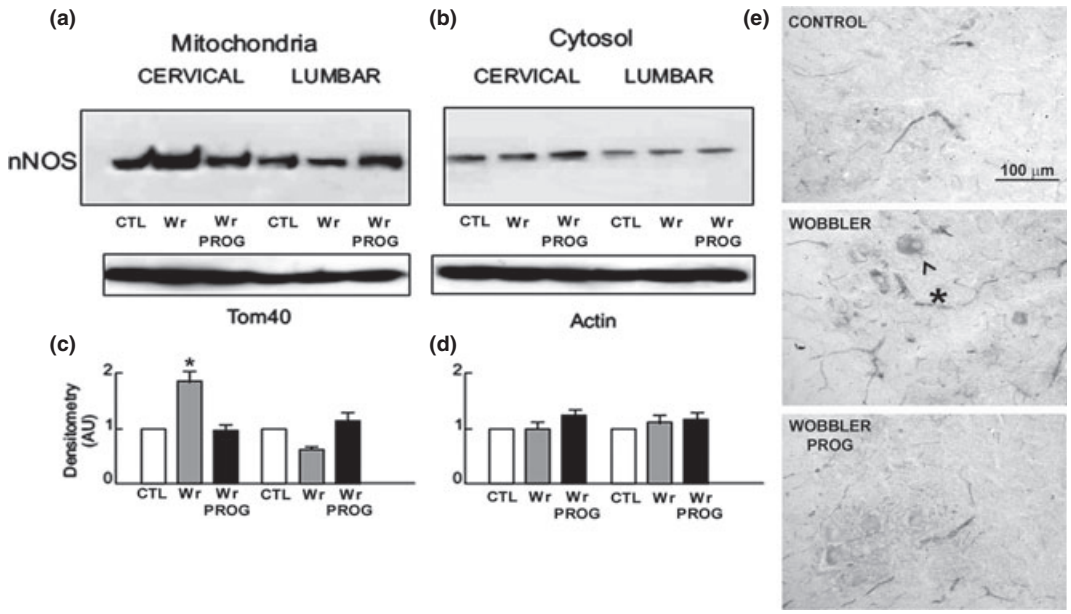

Fig. 2 Western blot analysis of nNOS protein in mitochondria and cytosol fraction from control mice, Wobbler mice and Wobbler mice receiving progesterone. (a) Increased signal for nNOS in the cervical, but not the lumbar region of the spinal cord, from Wobbler mice (Wr) compared with control mice (CTL). Progesterone reduced nNOS signal intensity in Wobbler cervical cord (Wr PROG). (b) Unchanged cytosolic nNOS signal in the cervical and lumbar regions of Wobbler compared with control or progesterone-treated Wobbler. (c) Densitometric measurement (expressed in arbitrary units, AU) showed significantly higher nNOS protein content in Wobbler mitochondria from the cervical cord (*$p < 0.05$) compared with control or progesterone-treated Wobbler. (d) Densitometric analysis of cytosolic nNOS showed similar levels in the cervical or lumbar regions in the three groups studied. Data were obtained from three separate experiments, each one comprising a pool of 12 animals per group. (e) Stronger NADPH-diaphorase staining shown by Wobbler spinal cord (middle image) compared with control mouse or progesterone-treated Wobbler (upper and lower images, respectively). Arrowhead in the middle image points to a motoneuron, asterisk to probable endothelial cells. Microphotographs were taken from the cervical region of the spinal cord. Inside bar: 100 μm.

Table 1 Mitochondrial respiratory enzyme complexes in the cervical and lumbar regions of the spinal cord of control, Wobbler mice and Wobbler mice receiving progesterone treatment

Complex activity (% of control)	Control	Wobbler	Wobbler prog	Control	Wobbler	Wobbler prog
Complex I	100	83.87 ± 2.91 (*** vs. ctl)	108.8 ± 1.06 (*** vs. Wr)	100	83.55 ± 1.83 (*** vs. ctl)	82.07 ± 2.71
Complex II-III	100	89.81 ± 3.06 (** vs. ctl)	95.1 ± 2.1	100	98.62 ± 6.41	92.77 ± 2.48
Complex IV	100	88.76 ± 11.1	95.83 ± 4.6	100	105.1 ± 8.74	95.69 ± 3.02

Progesterone effects on the activity of respiratory chain components in Wobbler mice

Having demonstrated the presence of increased intramitochondrial nNOS in the cervical spinal cord of Wobbler mice, and its reversal by progesterone treatment, we studied the consequences that changes of nNOS could have on respiratory chain components. Table 1 shows that complex I activity, expressed as % from control mitochondria, was significantly reduced in the cervical and lumbar regions of the spinal cord of untreated Wobbler mice (cervical: 83.8 ± 2.9%; $p < 0.001$ vs. control; lumbar : 83.5 ± 1.8, $p < 0.001$ vs. control). Progesterone treatment returned complex I activity to normal (108.8 ± 1.0%; $p < 0.001$ vs. untreated Wobbler). In the lumbar region, no effect on complex I activity was shown in the Wobbler mice with progesterone treatment. Table 1 also presents data on complex II-III activity. Wobbler mitochondria from the cervical region showed significantly reduced activity (89.8 ± 3.0%, $p < 0.01$ vs. control) but progesterone was unable to recover it. No differences were found for complex II-III activity in the lumbar spinal cord between the three animals groups. Measurement of complex IV activity did not produce statistically significant differences between control, Wobbler and Wobbler plus progesterone groups in the cervical or lumbar spinal cord.

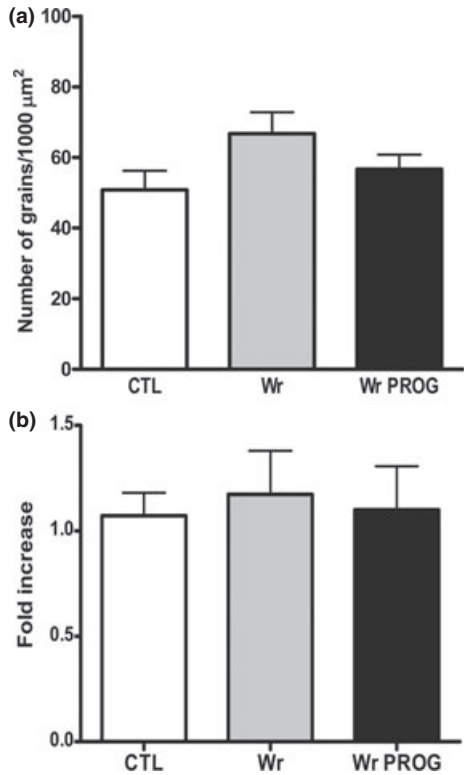

Fig. 3 (a) *In situ* hybridization (ISH) of nNOS mRNA in motoneurons from the cervical region of the spinal cords of CTL, Wr and Wr PROG mice. Results were expressed as no. grains per 1000 μm^2. Data were taken from four sections per mice and grains were counted from at least 12 motoneurons per section. $N = 5$ mice per group. (b) Quantitative real-time PCR of nNOS mRNA in the cervical region of the spinal cord of CTL, Wr and WR PROG groups. nNOS mRNA showed no statistical differences between the three groups by ISH or qPCR, suggesting that changes of nNOS protein due to the Wobbler mutation or progesterone treatment did not involve gene transcription. Results were the mean ± SEM of six animals per group.

Determination of nNOS mRNA by *in situ* hybridization and qPCR

Using ISH, the signal for nNOS mRNA was determined in ventral horn motoneurons from Lamina IX of the cervical region of the spinal cord. As shown in Fig. 3a, no significant differences in grain density (no. grains/mm^2) were obtained between control, Wobbler and Wobbler plus progesterone groups. We also performed real time PCR to determine the mRNA for nNOS in the cervical spinal cord. The results of this determination, expressed as fold-increase, did not reveal significant differences between control, Wobbler and Wobbler plus progesterone groups (Fig. 3b). These results suggest that gene transcription was not involved on the effects of the Wobbler mutation and progesterone treatment on mitochondrial mtNOS observed by western blot and nNOS observed by histochemistry for NADPH-diaphorase.

MnSOD enzyme activity and immunoreaction

Considering MnSOD as one of the most important cellular protective mechanisms against oxidative stress, we studied the effects of the Wobbler mutation and progesterone treatment on enzyme activity and immunostaining. We found that MnSOD activity of Wobblers was reduced by half compared with control and Wobbler plus progesterone (Fig. 4a, $p < 0.01$). We also found that the number of MnSOD immunopositive motoneurons were slightly, albeit significantly reduced, in the Wobblers versus controls (Fig. 4b, $^{*}p < 0.05$). In this case, progesterone treatment of Wobbler mice normalized the number of MnSOD-stained motoneurons (Fig. 4b, $^{\#}p < 0.05$). The photomicrographs of Fig. 4c show reduced MnSOD immunostaining of Wobbler ventral horn motoneurons compared with control, whereas progesterone treatment increased MnSOD labelling of the Wobbler.

Progesterone effects on APP immunocytochemistry

We determined the number of APP+ neurons as an index of axonal transport. Impaired traffic towards the pre-synaptic terminal, as it occurs in the Wobbler (Palmisano *et al.* 2011), allows the accumulation of organelles and macromolecules in the neuronal body, causing mitochondrial dysfunction. As expected for control tissues, the number of neurons showing APP immunostaining was very low (Fig. 5a). In contrast, numerous APP+ profiles were detected in the cervical spinal cord of steroid-naïve Wobblers ($p < 0.001$), whereas progesterone treatment of these mice significantly reduced the number of APP+ neurons ($p < 0.01$, Fig. 5a). Immunostaining (Fig. 5b) showed abundant APP+ profiles in Wobbler cervical spinal cord, which were reduced following progesterone treatment (Fig. 5b). Thus, this experiment suggested that progesterone enhanced the axonal transport of APP in Wobbler mice, based on the partial attenuation of APP accumulation observed in tissues from steroid-treated animals.

Discussion

This study demonstrated the effects of Wobbler mouse motoneuron degeneration and of progesterone treatment on mitochondrial-related parameters of the spinal cord. First, we found that the mitochondrial respiratory complex I of the cervical and lumbar regions and of complex II-III of the cervical cord showed a compromised activity in untreated Wobblers, in contrast to normal activities of cytochrome oxidase. Complex I activity of the cervical cord was normalized by progesterone. Second, we found high protein expression of NOS in mitochondria (mtNOS) but not cytosol of untreated Wobbler mice; in this study, the increased expression of mtNOS in Wobbler was normalized by progesterone treatment. Third, our data suggested that reduction of mitochondrial nNOS in progesterone-treated mice may be due to changes of the enzyme protein content

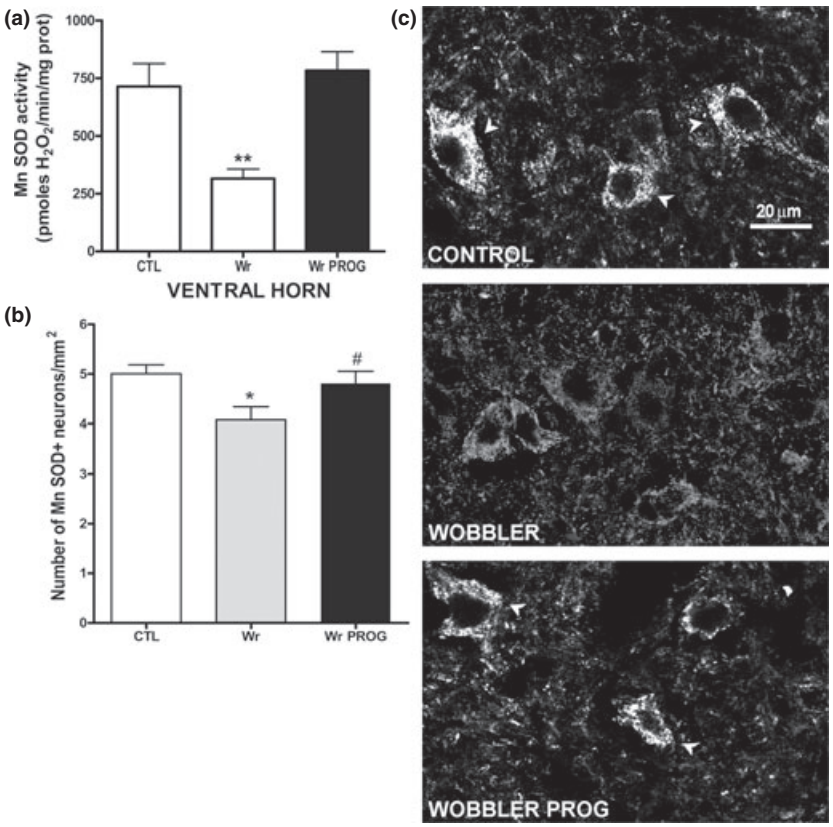

Fig. 4 Results of MnSOD activity (a) and of the number of MnSOD immunopositive motoneurons (b) in CTL, Wr and WR PROG groups. Wobbler mice showed decrease enzyme activity than CTL and WR PROG groups (a, $^{**}p < 0.01$) and decreased number of MnSOD+ motoneurons compared with CTL and Wr PROG groups (b, $^{*,\#}p < 0.05$). For statistical analysis, we used ANOVA and Newmam–Keuls *post hoc* test. Results of MnSOD activity represent the mean ± SEM of three separate experiments, each one comprising a pool of 12 animals per group. Immunostaining data were obtained from 10 sections per animal, $n = 7$ animals per group (mean ± SEM). (c) Images showing low MnSOD protein immunostaining in Wobbler (middle image) compared with control (upper image) and Wobbler PROG mice (lower image). Arrowheads in upper and lower photomicrographs point to MnSOD+ neurons. Microphotographs were taken from the cervical region of the spinal cord.Inside bar: 20 μm.

rather than on its transcriptional regulation, because levels of nNOS mRNA determined by ISH and qPCR were similar in untreated and progesterone-treated Wobblers. In confirmation of this finding, it has been shown that mtNOS is constitutively active (Finocchietto *et al.* 2009). Fourth, neurons labeled for APP were frequent in untreated Wobblers, suggesting decreased anterograde transport of APP to the terminal. Likewise, transport failure may facilitate accumulation of nNOS in the cell body and entry of this enzyme into the mitochondria. Progesterone treatment decreased APP-immunostaining, which may have some connection with the previously reported increase of axonal transport in the Wobbler mouse receiving progesterone (Gonzalez Deniselle *et al.* 2005). Fifth, enhancement of mitochondrial neurochemical parameters was accompanied by a reduced number of vacuolated motoneurons in the cervical region of the spinal cord of progesterone-treated Wobblers, indicating that mitochondrial abnormalities are linked to motoneuron degeneration. The finding that progesterone increased MnSOD activity and immunostaining in the Wobbler supports that progesterone's antioxidant effects can arrest neurodegeneration. It seems important to underlie that the above mentioned progesterone effects were exclusive of the cervical spinal cord, the prevalent site of motoneuron degeneration in the Wobbler mouse.

There is increasing evidence for the involvement of mitochondrial dysfunction in neurodegenerative diseases (reviewed in Schon and Przedborski 2011). Thus, changes of mitochondrial morphology, biochemistry and genetics have been implicated on the pathogenesis of Alzheimer' disease, Parkinson' disease and amyotrophic lateral sclerosis (Martin 2010). Our experimental data in the Wobbler mouse are in line with current hypothesis of strong mitochondrial involvement in this neurodegeneration model. As already mentioned, several reports have confirmed the impairment of mitochondrial respiration and function in the spinal cord or cerebral cortex of Wobbler mice (Xu *et al.* 2001; Dave *et al.* 2003a; Santoro *et al.* 2004). In our laboratory, we have

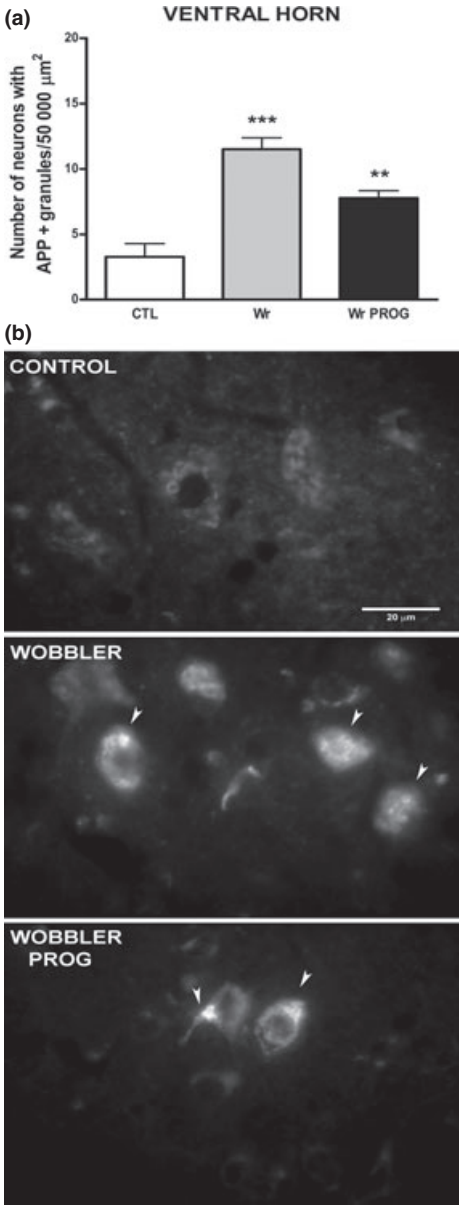

Fig. 5 (a) Number of motoneurons immunostained for amyloid precursor protein (APP) in CTL, Wr and Wr PROG groups. Wr showed significantly higher APP+ profiles than CTL (a, ***$p < 0.001$), whereas APP+ cells decreased in the Wr PROG group (a, **$p < 0.01$ vs. Wr). Ten sections of the spinal cord per animal were analyzed to determine the number of APP+ neurons using computed-assisted image analysis ($n = 10$ animals per experimental group). (b) Images showing high APP immunostaining in Wr (middle image) compared with weak immunostaining of control and moderate staining in a Wobbler + progesterone-treated mouse (upper and lower images, respectively). Arrowheads in middle and lower images point to APP+ neurons. Microphotographs were taken from the cervical region of the spinal cord. Inside bar: 20 μm.

previously demonstrated that degenerating mitochondria of the Wobbler occupied most of the cytoplasm and concentrated heavily around the nucleus, even altering nuclear shape (Gonzalez Deniselle *et al.* 2002). High magnification electron microscopy has shown that severely affected neurons of untreated Wobbler mice presented a disorganized mitochondrial ultrastructure, including oedematous matrix, cristolysis and loss of membrane integrity near the poles. We have also shown before that under progesterone treatment, some mitochondria reassumed a normal ultrastructure, together with a reduction of cytoplasmic vacuolation, enhanced axonal transport, increased expression of motoneuronal functional markers, increased muscle strength and prolonged animal survival (Gonzalez Deniselle *et al.* 2002, 2005, 2007). These reports are compatible with the present one supporting that the protective and beneficial roles of progesterone also applies to the mitochondrial dysfunction of the Wobbler. The present data, in addition to previous publications (Santoro *et al.* 2004; Gonzalez Deniselle *et al.* 2005; Fumagalli *et al.* 2006) have clearly shown that in the Wobbler mouse the target area for pathology is the cervical spinal cord. A possible rationale for sparing the lumbar motoneurons has been provided (Bastone *et al.* 2009). These authors have shown that over-expression of proteins involved in vesicle trafficking, together with proteins counteracting mitochondrial dysfunction can have neuroprotective effects on lumbar motor neurons of the Wobbler.

Of further interest is to consider the role of NOS and NO in the mitochondrial abnormalities of Wobbler mice. NO is considered a major regulator of the mitochondrial respiratory chain, and at high concentrations, it is driven to peroxinitrite generation ($ONOO^-$). The latter inhibits NADH dehydrogenase at the mitochondrial complex I by nitrosylation or nitration (Carreras *et al.* 2004). This mechanism could explain the reduced activity of complex I found in the isolated mitochondria of steroid-naïve Wobblers. Wobbler mice show increased nNOS in motoneurons, as first reported by Clowry and McHanwell (1996). The involvement of NO in Wobbler neuropathology is supported by studies showing that the NOS inhibitor 7-nitroindazole delays motor dysfunction and spinal motoneuron degeneration of Wobblers (Ikeda *et al.* 1998). We have previously published that the marked up-regulation of NOS activity in Wobblers was sensitive to progesterone. Employing 2- and 4-month-old Wobbler mice at the early and fully symptomatic stages of the disease (Gonzalez Deniselle 2004), we have shown that 18 days of progesterone treatment reduces the high number of NOS-active motoneurons and white matter astrocytes in 2-month-old Wobblers but is unable to change NOS-active motoneurons or astrocytes in older Wobbler mice. Since at that time we employed a histochemical reaction measuring NADPH-diaphorase activity, this finding is not in conflict with the present one, in which cytoplasmic NOS content measured by western blot was similar in control mice, untreated Wobblers and Wobblers receiving progesterone treatment. The cellular site(s) generating the excess NO should be a matter of further investigation, because not only

motoneurons but also astrocytes are a source of NO. In light of the early role that astrogliosis might have for neurodegeneration (Hantaz-Ambroise *et al.* 1994; Meyer *et al.* 2010), astrocyte-derived NO may be also implicated, because NO is a toxic gas that liberated from astrocytes damages motoneurons. In this regard, reactive astrocytes from a different ALS model produce nitric oxide and peroxynitrite, which cause mitochondrial damage in cultured neurons (Barbeito *et al.* 2004).

A significant effect of progesterone relates to its stimulatory effects on MnSOD. Within the mitochondria, MnSOD constitutes a leading defence against superoxide anion damage. This effect is due to generation of hydrogen peroxide from reactive oxygen species, which later decomposes due to the action of glutathione peroxidase and catalase (Pacher *et al.* 2007). Thus, it was highly rewarding to find out that progesterone re-established MnSOD activity and immunostaining. We suggest that degenerating motoneurons retain their capability to increase mitochondrial MnSOD in response to a protective stimulus, preventing the cellular damage provoked by reactive oxygen species originated in the untreated Wobbler motoneurons. However, even if progesterone shows an effect on MnSOD and complex I activity, the assumption of a direct antioxidant effect of progesterone remains as an attractive, but still unproven, hypothesis.

An unsolved question refers to the mechanism responsible for the increase level of mtNOS in the Wobbler. In this regard, we would like to postulate that decreased axonal transport of nNOS to the terminal could increase the enzyme levels in the cytoplasm, allowing for its entry into the mitochondria. Although the mechanisms of nNOS translocation are poorly defined, post-translational modifications of the enzyme such as phosphorylation or acylation have been proposed to control the import of this enzyme into the mitochondria (Finocchietto *et al.* 2009). A role of progesterone in these mechanisms remains to be established. We would like to conclude that mtNOS and NO are involved in motoneuron pathology of the Wobbler, suggesting that progesterone blockade of these factors may lead to neuroprotection. A closer look at the interaction between NO and progesterone may bring into light future therapeutic possibilities for Wobbler mouse neurodegeneration.

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