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PROTECTIVE EFFECTS OF THE NEUROSTEROID ALLOPREGNANOLONE IN A MOUSE MODEL OF SPONTANEOUS MOTONEURON DEGENERATION

Running title: Allopregnanolone and motoneuron degeneration.

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Highlights

1. Progesterone and allopregnanolone effects were evaluated in a model of motoneuron degeneration.
2. Both steroids increased protective signals such as pAKT and MnSOD in the spinal cord of Wobbler mice.
3. Both steroids decreased death signals such as p75NTR, pJNK, and NOS hyperactivity in Wobblers.
4. Increased BDNF and ChAT and enhanced muscle strength indicate steroid protection.
5. Allopregnanolone, a GABA_AR agonist, may become a therapeutic tool for motoneuron degeneration.

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a devastating disorder characterized by progressive death of motoneurons. The Wobbler (WR) mouse is a preclinical model sharing neuropathological similarities with human ALS. We have shown that progesterone (PROG) prevents the progression of motoneuron degeneration. We now studied if allopregnanolone (ALLO), a reduced metabolite of PROG endowed with gabaergic activity, also prevents WR neuropathology. Sixty-day old WRs remained untreated or received two steroid treatment regimens in order to evaluate the response of several parameters during early or prolonged steroid administration. ALLO was administered s.c. daily for 5 days (4mg/kg) or every other day for 32 days (3, 3 mg/kg), while another group of WRs received a 20 mg PROG pellet s.c. for 18 or 60 days. ALLO administration to WRs increased ALLO serum levels without changing PROG and 5 alpha dihydroprogesterone (5 α -DHP), whereas PROG treatment increased PROG, 5 α -DHP and ALLO. Untreated WRs showed higher basal levels of serum 5 α -DHP than controls. In the cervical spinal cord we studied markers of oxidative stress or associated to trophic responses. These included nitric oxide synthase (NOS) activity, motoneuron vacuolation, MnSOD immunoreactivity (IR), brain derived neurotrophic factor (BDNF) and TrkB mRNAs, p75 neurotrophin receptor (p75NTR) and, cell survival or death signals such as pAKT and the stress activated kinase JNK. Untreated WRs

showed a reduction of MnSOD-IR and BDNF/TrkB mRNAs, associated to high p75NTR in motoneurons, neuronal and glial NOS hyperactivity and neuronal vacuolation. Also, low pAKT, mainly in young WRs, and a high pJNK in the old stage characterized WR's spinal cord. Except for MnSOD and BDNF, these alterations were prevented by an acute ALLO treatment, while short-term PROG elevated MnSOD. Moreover, after chronic administration both steroids enhanced MnSOD-IR and BDNF mRNA, while attenuated pJNK and NOS in glial cells. Long-term PROG also increased pAKT and reduced neuronal NOS, parameters not modulated by chronic ALLO. Clinically, both steroids improved muscle performance. Thus, ALLO was able to reduce neuropathology in this model. Since high oxidative stress activates p75NTR and pJNK in neurodegeneration, steroid reduction of these molecules may provide adequate neuroprotection. These data yield the first evidence that ALLO, a gabaergic neuroactive steroid, brings neuroprotection in a model of motoneuron degeneration.

Keywords: allopregnanolone, progesterone, motoneuron degeneration, neuroprotection, wobbler mouse, amyotrophic lateral sclerosis, p75NTR, brain derived neurotrophic factor.

1. INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an adult fatal neurodegenerative disease, characterized by the selective and progressive death of both upper and lower motoneurons, leading to a progressive paralysis of voluntary muscles. Progesterone (PROG) therapy provides beneficial effects in animal models of ALS such as Wobbler (WR) mice and the SOD1 transgenic mice [1-6]. The neuroprotective effects of PROG and its reduced derivatives have also been shown in experimental ischemic stroke, brain and spinal cord trauma, diabetes mellitus, neuropathic pain and Alzheimer-like degeneration [7-11]. In the nervous system, the mechanism of action of PROG depends on binding to: nuclear receptors (PR), membrane progesterone receptors (mPR), the progesterone receptor membrane component 1 (PRMC1), sigma 1 receptors and, also, the modulation of neurotransmitter receptors by its ring A reduced derivative 3 α , 5 α -tetrahydroprogesterone or allopregnanolone (ALLO) [12-15]. Therefore, PROG metabolism into 5 α -dihydroprogesterone (5 α -DHP) and ALLO may play an important role in the neuroprotective mechanism [15]. In this regard, the neurosteroid ALLO, a positive modulator of GABA_A receptors, decreases neuropathology in animal models of degenerative disorders of the central and

peripheral nervous system such as Niemann-Pick, Alzheimer, Parkinson and diabetic neuropathy by increasing neurogenesis and oligodendrogenesis and reducing β -amyloid deposition, neuroinflammation and gliosis [8, 10, 11, 16-18]. ALLO also reduces excitotoxic damage of hippocampus and cerebral ischemic stroke [7, 9, 19]. *Consequently, ALLO may be a promising candidate for neuroprotective therapy in motoneuron degeneration. The potential benefit of using ALLO instead of PROG may reside on its protective effects at lower doses or even after a single administration as is demonstrated in Niemann-Pick type C disease [8, 20]. Furthermore, ALLO produces greater neuroprotection in traumatic brain injury or cerebral ischemia. Also, it has some properties not shared by PROG like the inhibition of the activity of the mitochondrial permeability transition pore, avoiding apoptosis [9, 19]. On the other hand, the ability of ALLO to interact with GABA_A receptors allows more accurately effects at the nervous tissue.*

WRs suffer a spontaneous mutation of the vacuolar/vesicular protein sorting 54 (Vps 54) gene [21] leading to motoneuron degeneration in motor cortex, brainstem and cervical spinal cord [22]. These alterations associate with increased oxidative stress, mitochondrial dysfunction, reduction of choline acetyl transferase (ChAT) and brain derived neurotrophic factor (BDNF) expression in motoneurons, impairment of slow axonal transport and gait disturbances [1, 2, 23, 24]. WRs and other models of ALS share similar pathological features, including intracellular ubiquitin inclusions, abnormal distribution of TDP-43 into the cytoplasm, cortical hyperexcitability and positive respond to Riluzole [5, 25-30]. Exogenous administration of PROG to WRs reduces morphological, molecular and functional abnormalities of motoneurons and glial cells and increases muscle strength and life span [2].

In the present investigation, we explored the possibility that ALLO, a PROG-reduced derivative, might also bring neuroprotection to the WR disease. Nitric oxide synthase (NOS) hyperactivity, and down-regulation of manganese superoxide dismutase (MnSOD) and BDNF characterized the motoneuron degenerative process in this model. Therefore, it seemed worthwhile to elucidate if ALLO treatment simultaneously modulated signs of motoneuron neuropathology, *NOS/NADPH diaphorase (NADPHd) activity*, BDNF receptors expression and signalling pathways related to cell survival or death such as pAKT or the c-Jun N-terminal kinases (JNK). Since BDNF effects are mediated by two types of cell surface receptors: the “prosurvival” TrkB family and the p75 neurotrophin receptor (p75NTR), expression of both receptors were also measured. The p75NTR is a member of the tumor necrosis factor (TNF) receptor superfamily, which could play 2 opposite roles: 1) “prosurvival” and 2) “prodegenerative” [31-33] and the release of its intracellular domain (icd) has

also been implicated in neurodegeneration. Our results provide evidence that ALLO *retards* several abnormalities of the WR spinal cord, suggesting alternative mechanisms for steroid neuroprotection.

2. MATERIALS AND METHODS

2.1 Experimental animals

Heterozygous male and female breeder mice (NFR background: NFR/wr) provided by the National Institutes of Health (Bethesda, MD, U.S.A.) were bred at the Instituto de Biología y Medicina Experimental animal facility. Animals were housed in group cages containing 2-3 symptomatic WR (wr/wr) with one non symptomatic mice (wr/+). This social interaction prolonged the life span and health status of WR mice [3]. The average lifespan of our colony is 7 months [34]. Animals were kept under conditions of controlled humidity and temperature (22° C) with lights on from 7:00 AM to 7:00 PM and fed with standard mice chow supplemented with a protein, mineral and vitamin nutrient (Ensure, Abbott). The Wobbler genotype (wr/wr) was identified employing an Alu I restriction polymorphism of a Cct4 amplification product. [35]. Cct4 diagnostic primers and restriction enzymes for genotyping were purchased from Promega Corporation (Madison, Wisconsin, USA). We employed 2 month-old symptomatic Wobbler mice in line with previous experiments [36]. At this stage, WR show ambulatory difficulties, muscle atrophy and forelimb flexion according to the criteria of Yung et al, 1994 [37]. Treatment was initiated at a stage when symptoms are progressing, in order to mimic the stage in which ALS patients are diagnosed [38]. Both sexes were used since neither the onset nor the progression of the disease correlated with sex [6, 34].

Based on previous reports [2, 39], two different treatment regimens were employed in order to evaluate parameters of early or late response to steroid administration: 1) oxidative stress parameters, mainly sensitive to short-term treatment and 2) neuronal functional parameters such as BDNF and ChAT, responding to long-term steroid administration. *Each steroid was administered following the procedure in which they were previously known to have neuroprotective effects [1, 2, 7, 11].* For PROG treatment, a single 20 mg PROG pellet was implanted under the skin of the neck at 60 days of age (Sigma, St. Louis, MO, USA) for 18 (short-term) or 60 days (long-term) under light isofluorane anesthesia [2, 39]. At 90 days of age, animals in the long-term group were re-implanted with a second PROG pellet until sacrifice. Sixty-day PROG treatment successfully restores the biceps weight of WR mice [34]. ALLO (Steraloids Inc., Newport, USA) treatment was administered s.c. daily for 5 days (short-term, 4mg/kg/day in 20%-cyclodextrin), in which the first dose was given i.p. This dose and duration of treatment protects neurons of the hippocampus after kainic acid

administration [7]. To evaluate long-lasting effects of ALLO, mainly at motor performance, a group of WR received a dose of 3.3 mg/kg/day of ALLO in 20%-cyclodextrin every other day for 32 days. At the time of sacrifice, animals were 78 (young WRs) or 120 (old WRs) day-old, respectively. The flowchart of Figure 1 summarizes the treatment paradigms.

For steroid measurements and Western blotting, animals were sacrificed by decapitation and cervical spinal cords were immediately removed following dorsal laminectomy, frozen on dry ice and kept at -80°C until used. For *in situ* hybridization (ISH) and histological staining procedures, mice were deeply anesthetized with a mixture of xylazine (6 mg/kg) and ketamine (75 mg/kg), perfused transcardially with 0.9% NaCl prepared in diethylpirocarbonate-treated water, followed by 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer pH 7.2. Thereafter, cervical spinal cords were immediately removed and postfixed for 2, 5 hs at 4°C in the same fixative. Then, the tissue was split into 2 segments: 1) a C₂₋₄ segment: immersed in graded ethanol, embedded in paraffin, and used for immunohistochemistry and cresyl violet staining for quantitation of vacuolated motoneurons; 2) a C₄₋₆ segment: cryoprotected by immersion in 20% sucrose overnight and kept frozen at -80°C until used for *in situ* hybridization and immunofluorescence.

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.

2.2 Steroid measurements

Levels of PROG and ALLO in serum and cervical spinal cord were measured at the end of the treatment by gas chromatography/mass spectrometry (GCMS) [40]. Data (mean $\pm$ SEM) were expressed as ng/ml serum and ng/g spinal cord, respectively.

2.3 *In situ* Hybridization (ISH)

ISH was carried out under RNase-free conditions following a previously published protocol [2]. In brief, 16 μm cryostat sections were fixed in 2% paraformaldehyde, washed in 0.5x sodium citrate/sodium chloride buffer (SCC), dried and acetylated. To detect BDNF and full length TrkB mRNA, we used 50 and 45 mer synthetic oligonucleotide probes complementary to: a) bp 650-699 of the coding region of mouse BDNF exon VIII

5'AGTTCAGTGCCTTTTGTCTATGCCCCTGCAGCCTTCCTTGGTGTAAC-CC-3' [41]; and b) bp 2781–2829 of the catalytic region of the full-length TrkB 5'GAAGGACTCTTCGGGTGATGCACTCTATCACCTCATTGTTTCG 3' (Oligos Etc, Inc., Wilsonville, OR, USA) [42]. Probes were end labeled with (³⁵S)dATP using the enzyme terminal transferase (Roche, Life Science, USA) as previously published [2]. After overnight hybridization at 42°C, sections were washed, dried, dipped into Kodak NTB-2 emulsion and exposed in the dark. Sections were then developed with Dektol (Kodak), fixed in Ektaflo fixer, counterstained with cresyl violet and coverslipped with Permount. A set of slides were hybridized in the presence of 20-fold excess of unlabeled probes. The specific signal was absent when tissue sections were preincubated with RNase (20 µg/ml, 30 min at 37°C) before ISH. *For quantitation of mRNA expression, the number of silver grains per cell was determined over medium and large motoneurons measuring >300 µm² located in the Lamina IX of the cervical spinal cord ventral horn [43]. Grain counting was performed by computer-assisted image analysis (Bioscan Optimas 6.02) and calculated after background subtraction [44]. Results were expressed as the mean grain density number of grains per 100 or 1000 µm² of soma ± S.E.M. Data from 30-40 neurons per animal corresponding to 6 sections (n= 7-9 mice per group) were combined to obtain a mean value per animal, and the animals were used as independent variables. Images were acquired at the same magnification using a digital camera Panasonic GP-KR222 connected to an Olympus BH2 microscope. To obtain film autoradiograms, sections were washed several times in SCC, dried after overnight hybridization at 42°C and then apposed against Kodak Biomax 35S-sensitive films for 24 h.*

2.4 NADPHd histochemistry

We employed a slight modification of the method of Vincent and Kimura [45] in order to detect NOS containing cells [46]. Briefly, cervical cord cryostat sections (16 µm) were postfixated by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.2 during 6 min at 4° C. After fixation, the sections were rinsed twice in phosphate buffered saline (PBS) and incubated in a solution of 0.1 M Tris-HCl buffer pH 7.4 containing 0.3% Triton X-100, 0.2 mg/ml of nitroblue tetrazolium (Sigma, St. Louis, MO, USA), 2.7 mg/ml l-malic acid (Sigma), and 1 mg/ml of β-NADPH (Sigma) in the dark during 90 min at 37°C [39]. Sections were then washed in PBS, dehydrated briefly in ethanol, dried and coverslipped with Permount.

2.5 Determination of vacuolated motoneurons

Paraffin sections (5µm) were stained with cresyl violet for light microscopy and quantitation of vacuolated cells. To recognize cytoplasmic vacuolation, we quantitated the number of cells showing vacuoles with a diameter >3µm in the cytoplasm [47]. The number of vacuolated motoneurons was quantified in 10 sections per animal (7-9 animals per group) in ventral horn using a computerized image analysis system (Bioscan Optimas 6.02) as previously published [3].

2.6 Immunoreaction for MnSOD, ChAT and p75NTR icd

For immunohistochemistry, paraffin sections from C₂-C₄ segments were rinsed in 0.01M phosphate buffer containing 0.14 M NaCl (PBS) and exposed to 0.3% H₂O₂ in methanol to block endogenous peroxidase. Sections were incubated with a goat anti-ChAT (1/200, AB144P-Millipore) or a rabbit anti-MnSOD polyclonal antibody (1/250, Stressgen) 4°C overnight after preincubation in 10% rabbit or goat serum, respectively. After washing, sections were incubated with a biotinylated rabbit anti-goat (ChAT) or goat anti-rabbit (MnSOD) secondary antibody (1/200 dilution, 60 min) and then with a 1/100 dilution of the ABC complex in PBS (ABC kit, Vector Labs, CA). The peroxidase activity was revealed using diaminobenzidine (DAB, 0.25 mg/ml) as substrate in the presence of 0.01% H₂O₂ in the dark. The number of immunoreactive ChAT or MnSOD positive cells was quantified in 10 sections per animal (7-9 animals per group) in ventral horn using a computerized image analysis system (Bioscan Optimas 6.02). Digital images (digital camera Panasonic GP-KR222 connected to an Olympus BH2 microscope) of tissue sections containing the spinal cord were displayed on the video screen under identical lighting conditions and grey-scale threshold.

For immunofluorescence, C₄-C₆ segments were used. Sixteen µm cryostat sections were rinsed in 0.1% Triton in PBS and then preincubated with 3% goat serum (p75NTR icd). Thereafter, we used an overnight incubation at 4°C with the rabbit polyclonal p75NTR icd antibody (1/500; G323A, Promega-Immunogen human p75 cytoplasmic domain) in 2% goat serum, and 1% Triton- X100 in PBS. After washing, sections were incubated with a goat anti-rabbit p75NTR icd Alexa Fluor 488 (1/1000 dilution). Sections were washed, dried, mounted with Fluoromont and examined under a Nikon Eclipse E 800 confocal scanning laser microscope equipped with Nikon 11691 photographic equipment. Digital images were taken under identical conditions. Cells were counted in greyscale images in 6-8 sections from 7-9 animals per experimental group.

In order to quantify immunoreactive cells, we set up a grey-scale threshold for positive cell area, and within this threshold, neurons were considered ChAT+, MnSOD+

or p75NTR icd+ cells according to previous reports using Optimas 6.02 software, [44, 48, 49]. Non-specific staining was assessed in the absence of primary antibody. Results were expressed as either the percentage of immunoreactive cells over the total number, or the number (mean $\pm$ S.E.M.) of cells/area (mm^2 or $10\,000\,\mu\text{m}^2$) in the ventral horn [3].

2.7 Double immunofluorescence p75NTR icd/ChAT confocal microscopy analysis

To localize p75NTR icd in ChAT immunoreactive motoneurons, 30 μm cryostat sections were incubated overnight at 4°C with a goat polyclonal ChAT antibody (1/200 dilution, Millipore) in 1% BSA, and 0.5% Triton- X100 in PBS after preincubation in 3%BSA. Then, sections were incubated with a donkey anti-goat IgG conjugated to Alexa Red 555 (1/1000 dilution, Invitrogen, Molecular Probes). After several washes in PBS, sections were incubated overnight at 4°C with the above-mentioned p75NTR icd rabbit polyclonal antibody (1/500 dilution) in 1% BSA and 0.5% Triton- X100 in PBS followed by goat anti-rabbit IgG conjugated to Alexa Green 488 (1/1000 dilution, Invitrogen, Molecular Probes, Eugene, OR, USA). Thereafter, sections were washed, mounted with Fluoromount-G (0100-01, SouthernBiotech), and examined under a Nikon Eclipse E 800 confocal scanning laser microscope equipped with Nikon 11691 photographic equipment. Non-specific staining was assessed in the absence of primary antibody. Since immunofluorescence for p75NTR icd showed a subcellular localization, neuronal bodies were scanned for cytoplasmic aggregates in at least 6 planes along the z axis. Confocal images were saved and further analyzed by a computerized image analysis system equipped with Optimas 6.0 software. We analyzed double-labeled p75NTR icd+/ChAT+ neurons in the ventral horn. The percentage of p75NTR icd+/ChAT+ neurons (mean $\pm$ SEM) was statistically compared between the control, WR, WR+PROG and WR+ALLO groups.

2.8 Western blot

Homogenates comprising the ventral horns were prepared by sonication in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na_3VO_4 , and 1% Triton 100, pH 7.4) containing a protease inhibitor cocktail (Roche Diagnostics) and the phosphatase inhibitors 10 mM NaF and 20 mM β -glycerolphosphate [50]. A total of 20 mg of protein was separated in 10% SDS-PAGE in Tris-glycine electrophoresis buffer at 120 V for 90 min. Proteins from gels were transferred onto PVDF membranes (Bio-Rad), and the membranes were blocked with TBS-T (20 mmol/l Tris, pH 7.5; 150 mmol/l NaCl; and 0.1% Tween-

20) containing 5% fat-free milk for 1 h. Blocked membranes were incubated with the primary antibody in TBS-T containing 5% fat-free milk at 4°C overnight. The following antibodies were used: rabbit anti-phospho AKT Ser 473 (1:2000, # 9271, Cell Signaling), rabbit anti-AKT pan (1:2000, # 4685, Cell Signaling), mouse monoclonal anti-phospho JNK (1:500, #6254, Santa Cruz), and actin (1:1000, #sc1616, Santa Cruz Biotechnology) [50]. Immunoblots were then washed with TBS-T three times and incubated at room temperature for 1 h with their respective HRP-conjugated secondary antibodies (1:5000, GE Healthcare Life Sciences, Buenos Aires, Argentina). Chemiluminescence was detected with the ECL system (GE Healthcare Life Sciences) and exposure to hyperfilm (GE Healthcare Life Sciences). Actin was used as a loading control. Quantification of bands was performed by digital image analysis using a Hewlett-Packard scanner and analyzed by Image J software (NIH, USA). Results were derived from 3 independent experiments ($n \geq 9$) and expressed as percentage of control in every experiment.

2.9 Motor behaviour analysis

Motor performance was studied by subjecting mice to a motor grip strength test [34]. In this test, the animals were allowed to hold by the forelimbs onto 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. *In order to assess the rate of variation in the time spent by the animals hanging from the rope, we calculated the ratio between the last and the first day of treatment.*

These measurements were performed weekly and values obtained from 12-15 animals per group were pooled for statistical analysis. All data were reported as mean $\pm$ SEM. WR mice had no hindlimb deformity during this study.

2.10 Statistical analysis

All results are expressed as mean $\pm$ S.E.M. Data were analyzed by one or two way ANOVA followed by the post-hoc Newman-Keuls or Bonferroni tests. Statistical analyses were performed with Prism 4 GraphPad software (San Diego, CA, USA). Significance was set at $p < 0.05$.

3. RESULTS

3.1 Steroid concentration

Two-way ANOVA (treatment x steroid) revealed a significant interaction between steroid treatment and steroid concentration ($p < 0.001$). *Bonferroni post-hoc test indicated a significant increase of basal levels of serum 5 α -DHP in WRs ($p < 0.05$), while it showed no difference in basal concentrations of PROG and ALLO between WR and control animals (Table 1).* PROG-treated-WRs during 18 days showed a 13-fold increase in the circulating levels of PROG ($p < 0.01$) and an 8.6 fold-change in ALLO ($p < 0.01$) compared to untreated WRs, while a non-significant increase was found for 5 α -DHP. ALLO-treated WRs during 5 days showed a 35-fold change in ALLO serum levels compared to untreated WRs, while it showed a 2.7-fold change compared to WRs receiving PROG ($p < 0.001$ vs WR; $p < 0.01$ vs WR PROG). In the ALLO-treated group, PROG and 5 α -DHP remained unchanged (NS vs WR) and were significantly lower than their PROG-treated counterparts ($p < 0.01$ vs WR PROG).

With regards to spinal cord steroid levels, PROG administration in WRs yielded a 61-fold higher levels in its tissue concentration ($p < 0.001$ vs WR) with a non-significant increase of ALLO concentration (Table 1). ALLO spinal cord levels experienced a 76-fold increase after its administration to WRs" ($p < 0.01$ vs WR, Table 1). No differences were found for PROG or ALLO levels in controls compared to WRs (NS vs WR).

3.2 ALLO effects on NADPHd activity, motoneuron vacuolation and MnSOD immunoreaction

In WR mice, high levels of NO impair mitochondrial function and damage motoneurons, leading to cytoplasmic vacuolation and generation of free radicals [1, 39]. Previous data have shown that enhanced NOS/NADPHd activity and increased intra-mitochondrial NOS expression are early markers of the WR disease. Here we studied the response of NOS/NADPHd activity in neurons and glial cells after short- and long-term administration of PROG or ALLO. The activity for NOS/NADPHd in neurons from the ventral horn, expressed as % of control activity, was 300% higher in young and 200% higher in old WRs vs control mice (Fig. 2A: young WRs, $p < 0.001$; Fig. 2E: old WRs, $p < 0.01$ vs. age-matched controls). NOS/NADPHd activity in young WRs was significantly reduced to nearly 160-180% after short-term PROG or short-term ALLO (Fig.2A: WR+short-term PROG, $p < 0.05$; WR+short-term ALLO, $p < 0.01$ vs. untreated

young WRs). WRs receiving long-term PROG showed NOS/NADPHd neuronal activity similar to control level (Fig 2E: $p < 0.01$ vs old WRs). However, no differences were found in WRs after long-term ALLO in comparison to old WRs (Fig 2E: $p > 0.05$ vs old WRs). Figures 2C and 2G show representative images of high NOS/NADPHd activity in ventral horn neurons of untreated WRs compared to the low activity in control, WR PROG and WR ALLO motoneurons.

Considering that glial cells are also NO producers, we next determined the number of NOS/NADPHd-positive glial cells in the ventrolateral funiculus (VLF) of the white matter. We found a higher number of NOS/NADPHd active glial cells in young and old WRs than age-matched controls (Fig. 2 B and F, young and old WRs: $p < 0.001$ vs controls). A significant reduction was demonstrated for both steroids independently of treatment duration (WRs+short-term PROG or ALLO: $p < 0.001$; WRs+long-term PROG or ALLO: $p < 0.05$ vs their age-matched WRs). The images of Figures 2D and H show the strong reaction for NADPHd in glial cells from WRs and the reduction after short (D) and long (H) steroid treatments.

In agreement with previous results, we found 30% and 10% of vacuolated motoneurons in the total ventral horn in young and old WRs, but none were found in controls (Fig. 3 A, B). However, a significant reduction of this parameter followed both protocols of PROG (Fig. 3 A, B: WR+short-term PROG: 15.7%, $p < 0.001$ or long-term PROG: 4.2%, $p < 0.001$ vs age-matched WRs) or ALLO (WR+short-term ALLO: 20.5% or long-term ALLO: 4.8%, $p < 0.01$ vs. their untreated counterparts). Figures 3C and D show several vacuolated motoneurons in the ventral horn of WRs (white arrowheads), which were absent in controls or scarcely present in steroid-treated WRs.

In addition to high levels of NO [1, 3], damaged motoneurons show low levels of the mitochondrial and antioxidant enzyme MnSOD. Consequently, we next analyzed the effects of both steroid regimens on MnSOD immunostaining. Untreated young and old WRs showed a reduction in the % of MnSOD immunoreactive neurons in ventral horn vs. their age-matched controls (Fig. 3E and 3F- young: 35.5% and old WRs: 8.9%; $p < 0.001$ vs young: 77.4% and old controls: 47%). Increased % of MnSOD neurons was obtained in WRs after long-term PROG (Fig 3F: 28.2%, $p < 0.05$ vs old WRs) or ALLO administration (Fig.3F: 54.4%, $p < 0.01$ vs old WRs). In contrast to long-lasting protocols, only short-term PROG elevated the % of MnSOD neurons in the WR's ventral horn (Fig.3E: 50.3%, $p < 0.01$ vs young WRs). Moreover, the values obtained after acute ALLO were lower than its PROG-treated counterpart ($p < 0.05$ vs WR PROG). Figures 3G (short-term) and H (long-term) show very low MnSOD staining in ventral horn neurons from WRs, and enhanced immunoreaction after long-term PROG or ALLO

administration. This finding indicates that ALLO's antioxidant capacity required the prolong regimen of administration.

3.3 Increase in ChAT immunostaining after long-term ALLO

Unlike the effect on vacuolated motoneurons, short-term steroid protocols did not modulate the immunostaining for ChAT in motoneurons from WRs (Fig.4A). Old WRs showed a significant reduction in the number of ChAT+ neurons in comparison to old controls (-33%, $p<0.01$). However, ChAT immunostaining was significantly improved after long-term administration of PROG (+46%, $p<0.001$) or ALLO (+40%, $p<0.01$ vs. age-matched WRs; Fig. 4B). The images of Figure 4C point out the enhanced ChAT immunoreaction in chronically PROG or ALLO-treated WRs compared to old WRs.

3.4 ALLO effect on BDNF and TrkB expression

Having demonstrated steroid effects on vacuolation and ChAT, we next studied if ALLO also modulated BDNF mRNA in motoneurons. In agreement with previous work, BDNF mRNA was reduced in young and old WRs vs controls (Fig.5A, B: $p<0.05$) [49]. Similar to ChAT, only long-lasting protocols of PROG or ALLO restored BDNF mRNA levels to control values (WR+long-term PROG and WR+long-term ALLO: $p<0.05$ vs old WRs, Fig.5B). Film autoradiograms and bright field images of individual motoneurons clearly indicated the decreased BDNF mRNA expression and the positive steroid effect after long-term treatment (Fig. 5C and D).

BDNF signalling through the high affinity neurotrophic receptor TrkB is associated with trophic responses. Therefore, we studied the expression of TrkB mRNA and its modulation by steroid treatment. As shown in Figures 5E and F, TrkB mRNA was reduced in motoneurons from young and old WRs (Fig. 5E: - 40%, $p<0.001$ and Fig 5F: - 30%, $p<0.05$ vs their age matched controls), while both PROG and ALLO (short- and long-term) administration elevated this parameter to control values (Fig 5E: WR+short-term PROG and WR+short-term ALLO: $p<0.001$, and Fig 5F: WR+long-term PROG, $p<0.01$; WR+long-term ALLO, $p<0.05$ vs age-matched WRs).

3.5 ALLO effect on the p75NTR icd

In addition to TrkB, BDNF also interacts with the low affinity p75NTR. We analyzed p75NTR icd+ cells in the ventral horn and double-labeled p75NTR icd+/ChAT+ cells in the same region. P75NTR immunoreaction showed an opposite

response compared to BDNF and TrkB and similar to NOS/NADPHd activity. Thus, p75NTR icd+ cells/unit area were markedly up-regulated by nearly 100% in young and old WRs (Fig 6-upper graphs: $p < 0.001$ vs their age-matched controls). A significant reduction was obtained after both steroid regimens (Fig 6-upper graphs: WR+short-term PROG and WR+short-term ALLO, $p < 0.001$ vs. young WRs; WR+long-term PROG: $p < 0.01$; WR+long-term ALLO: $p < 0.05$ vs. old WRs). Figures 6 A-D show a strong immunoreaction for p75NTR icd in cells of the ventral and dorsal horns from old WRs (Fig 6B) compared to age-matched controls (Fig 6A), WR+long-term PROG (Fig 6C) and WR+long-term ALLO (Fig.6D). Figures 6 E-H show cells located in the region of the white square at high magnification. Double immunofluorescence for p75NTR icd/ChAT revealed higher levels of colocalization in young and old WRs, in contrast to observations made in controls of both ages (Fig 7 A, B- $p < 0.05$ vs their age-matched controls). Short or prolonged administration of ALLO decreased the percentage of p75NTR icd/ChAT positive motoneurons to control values (Fig 7 A, B: WR+short-term ALLO $p < 0.05$; WR+long-term ALLO, $p < 0.01$ vs. age-matched WRs). However, only long-term PROG reduced double-labelled motoneurons (Fig 7: WR+short-term PROG $p > 0.05$; WR+long-term PROG $p < 0.05$ vs age-matched WRs).

3.6 Modulation of pAKT and pJNK by ALLO

Because neurodegeneration is associated with death pathways involving p75NTR, we studied changes of the death signal JNK and the survival factor AKT in steroid-naïve WRs and their modulation by steroid treatment. The phosphorylation of JNK isoforms 1 and 2/3 was 1.4-fold higher in the ventral horn from old WRs compared to old controls (Fig. 8B, $p < 0.05$). In contrast, a non-significant increase was demonstrated in young WRs (Fig8A, $p > 0.05$ vs young controls). PROG and ALLO significantly reduced the high levels of pJNK to control values only after the long-term administration (Fig. 8B: $p < 0.05$ vs old WRs), whereas a non-significant reduction of this factor followed both short-term steroid treatments (Fig.8A). Conversely, young but not old WRs showed a 40% reduction of pAKT in the ventral horn compared to young controls (Fig8C: $p < 0.05$), while old WRs showed values similar to old controls (Fig 8D). Short-term administration of PROG and ALLO significantly increased pAKT in the WR's ventral horn (Fig.8C: $p < 0.05$ vs young WRs), whereas only long-term PROG treatment was effective (Fig.8D: $p < 0.05$ vs old WRs). In contrast, a slight but non-significant elevation followed long-term ALLO (Fig 8D: $p > 0.05$ vs old WRs). Table 2 gives an overview of the effects of ALLO and PROG administration on the reported spinal cord parameters in the WR mice spinal cord.

3.7 Effect of PROG and ALLO treatments on muscle strength

The test of muscle function measured the hanging time from a horizontal rope between untreated WRs and WR+long-term PROG or WR+long-term ALLO. The ratio of the latency to fall between the last day and the first day of treatment was calculated *in order to assess the rate of variation in the time spent by the animals hanging from the rope (detailed in Supplementary Table 1). This ratio was significantly higher in WRs+long-term PROG ($p<0.05$) and WRs+long-term ALLO ($p<0.01$) compared to old WRs (Fig. 9).*

4. DISCUSSION

The present study demonstrated that ALLO showed trophic effects and modulated mediators of oxidative stress and death/survival signals in the degenerating spinal cord of the WR mouse. The exogenous administration of ALLO increased its serum levels, without altering 5 α -DHP or PROG levels. Instead, PROG treatment increased the levels of its metabolites, which makes it difficult to appraise the role of each neuroprotectant. We showed that ALLO provided neuroprotection by reducing glial NOS/NADPHd hyperactivity, motoneuron vacuolation, p75NTR icd immunoreactivity in motoneurons and death signals (pJNK). Simultaneously, ALLO showed positive modulation of the antioxidant enzyme MnSOD, the low levels of the mRNAs for BDNF and TrkB, and ChAT immunostaining. Prolonged treatment with this neurosteroid also slowed the deterioration of muscle performance. *On the other hand, PROG results confirmed previous findings showing that short-term PROG reduced vacuolation, hyperactivity of NOS/NADPHd in neurons and glial cells and increased MnSOD immunoreactivity in WRs. Also, long-term PROG increased ChAT and BDNF in motoneurons and improved muscle strength of WR mice [1, 2, 39].*

Using specialized methodology, high serum levels of 5 α -DHP associated to a slight increase of PROG and ALLO were shown in untreated WRs in line with previous reports. High PROG concentrations were also demonstrated in serum from ALS patients [51, 52]. The adrenal glands may contribute to this elevation in WRs, since: a) basal and stress levels of circulating corticosterone are higher in WR mice suggesting adrenal hyperfunction, b) the adrenal zona fasciculata shows focal regions of hypertrophied cells and c) adrenal glands from WRs show high levels of PROG [52]. Furthermore, increased levels of 5 α -DHP in sera from WRs suggest a high 5 α -reductase activity in peripheral tissues. Because 13-fold higher levels of circulating PROG after treatment were associated to an increase in 5 α -DHP and ALLO, the three steroids may maintain neuronal homeostasis and contribute to neuroprotection [9]. Regarding ALLO, administration to WRs did not change the circulating levels of its progenitor steroids, implying that neurotrophic effects of ALLO may directly modulate its downstream targets, even when the possibility exists for a back conversion of ALLO into 5 α -DHP, a ligand for the classical PR [13, 15]. These data suggest the existence of several mediators for ALLO protective effects, since ALLO (1) regulates the alpha (α) and delta (δ) GABA_A receptor subunits and the activity of this neurotransmitter receptor, (2) binds to the pregnane X receptor (PXR) or interacts with (3) membrane PR (mPR) and (4) sigma1 receptors. Hypothetically, these molecules may be involved

in ALLO neuroprotection [53]. Furthermore, an antiglutamatergic effect of ALLO on NMDA receptors may also contribute to its protective action. This mechanism could decrease intracellular Ca^{++} and, in consequence, Ca^{++} -dependant activation of enzymes such as nNOS [54]. *Oxidative stress plays a key role in the pathogenesis of ALS and the WR's disease. It predominates in neurons at the early symptomatic stage and is less prominent at the late stage of the disease [39]. An increased generation of free radicals, 4-hydroxynonenal and high activity of NOS are indicators of oxidative stress in the spinal cord of WRs [1, 55]. Likewise, the attenuation of motoneuron degeneration by antioxidant agents, nitric oxide inhibitors, the antioxidant steroid U-74389F and edaravone, a free radical scavenger, also support the importance of oxidative stress in WRs motoneurons [56-58]. Our results showed that NOS/NADPHd-active motoneurons and glial cells were about 3-fold higher in young WRs than controls, coincided with the main period of motoneuron death. These results support the contribution of oxidative stress and mitochondrial dysfunction in the early stage. In this regard, the reduction of neuronal vacuolation and NOS/NADPHd activity by PROG and ALLO may be a mechanism of neuroprotection. Vacuolation is a cell death mechanism related to oxidative stress, which predominates in neurons in the early symptomatic stage of WRs as well as NOS/NADPHd hyperactivity [59-61]. Concerning ALLO, it decreased glial and neuronal NOS activity, whereas the effect was exclusively neuronal after short-term administration. On the other hand, both PROG treatment protocols significantly reduced NOS/NADPHd activity and the percentage of vacuolated cells. It is possible that PROG was acting through a genomic mechanism, as PR is expressed by rodent motoneurons [9]. However, ALLO might regulate nNOS activity through a non-genomic mechanism on GABA_A or glutamate receptors. For instance, 4 day application of ALLO to developing neuronal cells increases $\alpha 4$ GABA_A receptor subunit [62, 63]. Likewise, short-term ALLO might increase δ GABA_A receptor subunit, which is important for neurosteroid modulation of tonic inhibition, a major mechanism to control hyperexcitability of motoneurons [64-67]. Furthermore, it is also possible that short-term ALLO blocks glutamate excitotoxicity and, consequently, Ca^{++} dependent activation of nNOS in motoneurons [7, 54]. The lack of NOS/NADPHd reduction in WR motoneurons but not in glial cells after long-term ALLO also suggests that neurosteroid neuroprotection depends on the cell type or isoform of NOS.*

We also showed that, similarly to PROG, prolonged but not short ALLO treatment, significantly restored ChAT immunoreaction and BDNF mRNA in motoneurons. This PROG effect may be PR-mediated, as coincubation of PROG with the PR-antagonist RU-486 reduced the levels of ChAT in embryonic motoneurons [68, 69]. However, ALLO effects on ChAT are probably PR-independent and due to the

early reduction of NO production and the modulation of neurotransmitters, a mechanism already reported for traumatic brain injury in rats [53, 70].

Effects on BDNF are closely related to p75NTR and TrkB receptors. The increase of p75NTR in the spinal cord from young WRs overlapped with high levels of intracellular oxidative stress, which might activate p75NTR in the absence of ligands in the early stage of the WR disease [71]. Likewise, neurotrophins may be modified under conditions of high oxidative stress, making them refractory to cleavage. The imbalance in favour of pro-neurotrophins may change signalling pathways towards cell death. In young WRs, downstream signalling of p75NTR and TrkB pathways were markedly altered, probably resulting in reduced pAKT. Thereafter, increased death signals, such as high pJNK, characterized the old stage. The precursor molecule proBDNF might play a role in this process by stimulating Ca^{++} uptake through p75NTR, reducing pAKT and enhancing pJNK [72, 73]. Here, low pAKT expression in the early stage of the WR disease was accompanied by NOS/NADPHd hyperactivity and low MnSOD, suggesting a link between increased NOS activity, decreased antioxidant capacity, AKT dephosphorylation and selective motoneuronal degeneration. The phosphorylation of AKT is important for cell survival, and is also recognized as a primary mediator of the downstream effects of phosphatidylinositol 3-Kinase (PI3K) [74]. *Conversely, pAKT did not decrease in the ventral horn from old WRs. Preliminary results suggest that the lack of differences between old WR and control mice was due to increased expression of pAKT by glial cells, possibly to afford neuroprotection at late stages of the disease.* In young WRs, MnSOD did not show a response after short-term ALLO treatment, while short-term PROG was effective. However, the degeneration pathway was partially prevented by this ALLO treatment protocol because it decreased p75NTR, motoneuron vacuolation, nNOS activity and increased the mRNA for TrkB and pAKT. Concerning the long-term protocols, both steroids up-regulated MnSOD, the mRNAs for BDNF and TrkB and reduced p75NTR in motoneurons, while only chronic PROG increased pAKT and reduced neuronal NOS/NADPHd activity. These data suggest that early reduction of oxidative stress by PROG, possibly by increasing the mitochondrial antioxidant capacity of MnSOD, contributed to pAKT modulation after long-term administration. Regarding pJNK, its phosphorylated form prevailed in old WRs, suggesting that this factor may be more sensitive to regulation by steroids after the long-lasting protocol or in the late stage. In fact, both PROG and ALLO reduced pJNK levels after their prolonged administration. Another possibility is that the reduction of pJNK in degenerating motoneurons may require decreased oxidative stress and an improved mitochondrial function.

Regarding pharmacological effects of ALLO after long-term administration on ChAT, BDNF or MnSOD in WR motoneurons, it is possible that this reduced-progesterone derivative activates gene expression through PXR or mPR, as mPR beta is expressed by rodent spinal cord motoneurons [9, 53].

This course of events suggests a connection between steroid effects on cell survival or death signals and steroid antioxidant properties. In this regard, PROG and ALLO rapidly affect neuronal function by modulation of membrane PR receptors or neurotransmitter receptors such as GABA_A, sigma 1 and NMDA. Literature reports support this possibility [4, 12, 75-77]. *However, the protective effect of each steroid might be cell and target specific. We suggest that PROG effects mainly depend on the activation of the classical PR since low amounts of ALLO was measured in tissue after PROG treatment. On the other hand, the effects of ALLO may rely on the subunit combination of GABA_A receptor prevailing after treatment. Furthermore, ALLO could also prevent neuronal vacuolation and NO production by blockage of glutamate release [78]. Neuroprotective effects of this reduced steroid may also depend on inhibition of apoptosis [53] and maintenance of mitochondrial membrane integrity [79]. These combined effects became protective for motoneurons, as shown by the concomitant increase of the cholinergic marker ChAT, the antioxidant enzyme MnSOD, BDNF and TrkB, reduction of pJNK and the enhanced motor performance of WRs receiving steroid treatment [2].*

5. CONCLUSIONS

The present results show that both PROG and ALLO modulated trophic molecules, survival signals and oxidative stress parameters in WR spinal cord, providing an adequate environment for neuroprotection. *It seems important to emphasize that both steroids recovered NOS, pAKT and neuronal vacuolation in the early stages of degeneration, suggesting that inhibition of oxidative stress plays a role in steroid action. Our data also suggest that chronic effects of ALLO and PROG rely on the up-regulation of BDNF expression, plus a reduction of pJNK. Therefore, different mechanisms of steroid action may be involved in motoneuron protection. Preclinical studies with ALLO may provide additional translational perspectives for motoneuron degenerative disorders.*

The authors report no conflict of interest.

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FIGURES

Figure 1: Time course for ALLO and PROG treatments in WR mice. **A:** a') WRs receiving short-term PROG treatment: WR PROG. a'') WRs receiving short-term ALLO treatment: WR ALLO. Animals were killed at 78 days of age: young WRs. **B:** b') WRs receiving long-term PROG treatment: WR PROG. b'') WRs receiving long-term ALLO treatment: WR ALLO. Animals were killed at 120 days of age: old WRs. WR: Wobbler; PROG: progesterone; ALLO: allopregnanolone

Figure 2: Both treatment protocols of PROG and ALLO decreased NOS/NADPH-diaphorase hyperactivity in neurons (ventral horn) and glial cells (VLF), except for ALLO in neurons in the long-term treatment.

A,E: Neurons positive for NOS/NADPHd activity in ventral horn expressed as % of control. **B,F:** Number of NOS/NADPHd positive glial cells in VLF/10000 μm^2 . WR vs Control- **A:** *** $p < 0.001$; **E:** ** $p < 0.01$; **B, F:** *** $p < 0.001$. WRs+short-term PROG vs WR- **A:** * $p < 0.05$; **B:** *** $p < 0.001$; WRs+long-term PROG vs WR- **E:** ** $p < 0.01$ and **F:** * $p < 0.05$. WRs+short-term ALLO vs WRs- **A:** ** $p < 0.01$; **B:** *** $p < 0.001$. WRs+long-term ALLO vs WRs- **E:** NS; **F:** * $p < 0.05$.

Results represent the mean $\pm$ S.E.M of 6 sections per animal. White arrows indicate NOS/NADPHd positive neurons. White arrowheads show cells with low NOS/NADPHd activity. Inside bar: 50 μm (**C, G**- ventral horn neurons); 20 μm (**D, H**- VLF-glial cells). NADPHd: NADPH diaphorase; VLF: ventrolateral funiculus; WR: Wobbler; PROG: progesterone; ALLO: allopregnanolone

Figure 3: Both PROG and ALLO treatment protocols reduced motoneuron vacuolation; PROG increased the low MnSOD immunoreactivity in ventral horn neurons in both protocols while ALLO only in the long-term protocol.

A-D: Motoneuron vacuolation was not observed in the ventral horn from controls (ND) while high % of vacuolated neurons was demonstrated in young and old WRs and scarce vacuolation after steroid treatment. **A, B:** WRs+short or long-term PROG: *** $p < 0.001$ and WRs+short or long-term ALLO ** $p < 0.01$ vs. age-matched WRs. **C, D:** Images showing intensely vacuolated motoneurons (white arrows) in untreated WRs but scarce vacuolation and neurons with normal appearance (white arrowheads) in steroid-treated WRs. **E, H:** Low % of MnSOD+ neurons in young and old WRs (*** $p < 0.001$ vs age-matched controls). **E:** High % of MnSOD+ neurons was shown in WR+short term PROG (* $p < 0.01$ vs young WRs and χ $p < 0.05$ vs. WR+short-term ALLO). WR+short-term ALLO did not modify MnSOD in WRs (NS). **F:** High % of MnSOD+ neurons in WRs+long-term PROG (* $p < 0.05$) and WRs+long-term ALLO (** $p < 0.01$) vs. age-matched WRs. **G, H:** Images showing cells with high immunoreactivity (white arrowheads) and cells with signal levels below threshold (white arrows). Inside bar: 20 μm . Results represent the mean $\pm$ S.E.M. ND: not detectable. MnSOD: manganese superoxide dismutase; WR: Wobbler; PROG: progesterone; ALLO: allopregnanolone

Figure 4: Long-term treatment with PROG and ALLO increased ChAT immunoreactivity in motoneurons from WR's ventral horn.

A,B: The number of ChAT+ cells/ mm^2 was reduced in old WRs (** $p < 0.01$) but not in young WRs (NS vs. age-matched controls) and it was increased in long-term PROG (** $p < 0.001$) and ALLO (** $p < 0.01$ vs. age-matched WRs). Results represent the mean $\pm$ S.E.M. **C:** Representative microscopy images of ChAT immunoreactive motoneurons in control, WR, WR PROG and WR ALLO corresponding to the long-term treatment.

Inside bar: 50 μ m. ChAT: choline acetyltransferase, WR: Wobbler; PROG: progesterone; ALLO: allopregnanolone

Figure 5: PROG and ALLO increased motoneuron expression of BDNF and TrkB mRNAs in WRs after the long-term treatment, while the short-term treatment only elevated TrkB mRNA.

A,B: Lower levels of BDNF mRNA (number of grains/100 μ m²) was demonstrated in motoneurons by *in situ* hybridization in young and old WRs (* p <0.05) than age-matched controls; while it increased (**B**) in WR+long-term PROG (& p <0.05) and WR+long-term ALLO (& p <0.05) vs. age-matched WRs. **C:** Low magnification images of film autoradiograms showing the expression of BDNF mRNA in cervical spinal cord. Circles indicate the region (Lamina IX) in which mRNA expression was analyzed. **D:** Light microscopy images of motoneurons located in lamina IX showing abundant grain deposits in control and WR + long-term PROG or WR + long-term ALLO treatments in contrast to the paucity of grains in old WRs. Inside bar: 20 μ m.

E, F: Lower levels of TrkB mRNA (number of grains/1000 μ m²) was shown in young (**E**, *** p <0.001) and old WRs (**F**, * p <0.05) than age-matched controls, while it increased after short-term PROG and ALLO (**E**, && p <0.001 vs young WRs) and after long-term PROG (**F**, && p <0.01) and WR+long-term ALLO (& p <0.05 vs. old WRs). Results represent the mean $\pm$ S.E.M. BDNF: brain derived neurotrophic factor; WR: Wobbler; PROG: progesterone; ALLO: allopregnanolone

Figure 6: Short and long-term PROG and ALLO treatments reduced the high number of p75NTR icd positive cells in the ventral horn from WRs.

High number of p75NTR icd+ cells/10000 μ m² in WRs vs age-matched controls (*** p <0.001) that decreased after short (&& p <0.001) and long-term steroid treatment (& p <0.05 vs age-matched untreated WRs). Results represent the mean $\pm$ S.E.M. **A-D:** low magnification of dorsal and ventral horns. **E-H:** Cells located in the region of the white square at high magnification corresponded to the ventral horn region. Inside bar: 50 and 15 μ m (left and right panel, respectively). **A,E:** old control; **B,F:** old WR; **C,G:** WR+long-term PROG; **D,H:** WR+long-term ALLO. WR: Wobbler; PROG: progesterone; ALLO: allopregnanolone

Figure 7: Long-term PROG and ALLO administration reduced the high % of p75NTR icd+/ChAT+ colocalization in motoneurons from WR's ventral horn, while only short-term treatment of ALLO was effective.

A, B: High % of p75NTR icd+/ChAT+ colocalization by immunofluorescence in young and old WRs (* p <0.05 vs age-matched controls) was reduced after short-term ALLO (& p <0.05), long-term PROG (& p <0.05) and long-term ALLO (& p <0.01 vs age-matched WRs). Results represent the mean $\pm$ S.E.M. **C:** Confocal images of ChAT+ cells (white arrows, red), p75NTRicd+ cells (white arrows, green) and p75NTRicd+/ChAT+ cells (white arrows, yellow) in the ventral horn of controls, WR, WR PROG and WR ALLO after long-term treatment. Inside bar: 50 μ m. ChAT: choline acetyltransferase; WR: Wobbler; PROG: progesterone; ALLO: allopregnanolone

Figure 8: Long-term PROG and ALLO re-establish pJNK levels, whereas short-term PROG and ALLO and only long-term PROG treatments re-established pAKT.

Data were quantified by densitometric analysis and corrected for actin as the loading control. Representative blots of pJNK/actin (**A, B**) and pAKT/AKT (**C, D**) expression are shown under each corresponding graph. Densitometric measurements (in arbitrary units, AU) were expressed as percentage of control. **B:** Higher levels of pJNK were found in old WRs than age-matched controls (* p <0.05), which reduced by long-term

PROG or ALLO administration ($^{\&p}<0.05$ vs untreated old WRs). **C:** Lower levels of pAKT were found in young WRs than their age-matched controls ($^{\&p}<0.05$) and increased after short-term PROG or ALLO treatments ($^{\&p}<0.05$ vs untreated young WRs). **D:** PROG but not ALLO significantly increased pAKT in WRs after long-term administration ($^{\&p}<0.05$ vs age-matched WRs). Results were expressed as the mean $\pm$ S.E.M. WR: Wobbler; PROG: progesterone; ALLO: allopregnanolone

Figure 9: Improvement of forelimb grip strength in WRs after long-term PROG and long-term ALLO.

Latency to fall in seconds expressed as the ratio between the end and day 1 of treatment in old WRs and WRs after long-term PROG or ALLO administration. Significant improvement in grip strength was seen in WR PROG ($^{\&p}<0.05$) and WR ALLO ($^{\&p}<0.01$ vs old Wobblers).

A SHORT-TERM TREATMENT

B LONG-TERM TREATMENT

NOS/NADPHd histochemistry

Short-term treatment

Long-term treatment

Motoneuron Vacuolation

MnSOD immunohistochemistry

ChAT Immunohistochemistry

BDNF mRNA

C

D

TrkB mRNA

p75NTR icd immunofluorescence in Ventral horn

p75NTR icd in ChAT immunoreactive motoneurons

C

pJNK **pAKT**

Grip Strength

TABLE 1: Progesterone (PROG), 5 α -dihydroprogesterone (5 α -DHP) and allopregnanolone (ALLO) levels in serum and spinal cord from young controls, young Wobblers (WR), WR PROG and WR ALLO

Results are expressed as mean $\pm$ S.E.M. Data were analyzed by two way

	SERUM (ng/ml)			CERVICAL SPINAL CORD (ng/g)	
	PROG	α -DHP	ALLO	PROG	ALLO
CONTROL	0.92 $\pm$ 0.32	46 $\pm$ 0.09	0.26 $\pm$ 0.05	2.22 $\pm$ 0.81	0.49 $\pm$ 0.10
WOBBLER	2.15 $\pm$ 0.47	57 $\pm$ 1.43	1.62 $\pm$ 0.72	3.04 $\pm$ 0.97	2.31 $\pm$ 0.53
WOBBLER PROG	27.97 $\pm$ 0.17 ##	51 $\pm$ 0.53	15.50 $\pm$ 7.91 ##	190.70 $\pm$ 32.30 ###	17.80 $\pm$ 1.18
WOBBLER ALLO	2.99 $\pm$ 1.12 **	38 $\pm$ 0.34	58.58 $\pm$ 5.6 ### / **	ND	179.6 $\pm$ 40.2 ### / ***

ANOVA and Bonferroni post-hoc test. P values: p<0.05 #; p<0.01 ##; p<0.001 ### vs WR; p<0.01 **; p<0.001 *** vs WR PROG. ND: not determined.

Table 2: Overview of the effects of progesterone (PROG) and allopregnanolone (ALLO)

treatment
s
in the
spinal
cord of
Wobblers
(WR)

Groups Factors	Location	WOBBLER		WOBBLER PROG		WOBBLER ALLO	
		Young	Old	Short	Long	Short	Long
NOS/NADPHd	Ventral horn neurons	↑↑	↑	↓	↓↓	↓	—
	glial cells in VL funiculus	↑↑	↑↑	↓	↓	↓	↓
Vacuolation	Ventral horn neurons	↑↑	↑	↓↓	↓↓	↓	↓
Mn SOD	Ventral horn neurons	↓↓	↓↓	↑	↑	—	↑↑
ChAT	Ventral horn motoneurons	—↓	↓↓	—	↑↑	—	↑↑
BDNF mRNA	Ventral horn motoneurons	↓	↓	—	↑	—	↑
TrKB mRNA	Ventral horn motoneurons	↓↓	↓↓	↑↑	↑↑	↑↑	↑↑
p75NTR icd	Ventral horn neurons	↑↑	↑↑	↓↓	↓↓	↓↓	↓↓
p75 NTR icd in ChAT+ motoneurons	Ventral horn motoneurons	↑	↑	—	↓	↓	↓
pJNK	Ventral horn	—↑	↑	—	↓	—	↓

pAKT	Ventral horn	↓ —	↑ ↑	↑ -↑
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