Sigma-1R Agonist Improves Motor Function and Motoneuron Survival in ALS Mice

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Published online: 31 August 2012
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Abstract Amyotrophic lateral sclerosis is a neurodegenerative disorder characterized by progressive weakness, muscle atrophy, and paralysis due to the loss of upper and lower motoneurons (MNs). Sigma-1 receptor (sigma-1R) activation promotes neuroprotection after ischemic and traumatic injuries to the central nervous system. We recently reported that sigma-1R agonist (PRE-084) improves the survival of MNs after root avulsion injury in rats. Moreover, a mutation of the sigma-1R leading to frontotemporal lobar degeneration/amyotrophic lateral sclerosis (ALS) was recently described in human patients. In the present study, we analyzed the potential therapeutic effect of the sigma-1R agonist (PRE-084) in the SOD1^{G93A} mouse model of ALS. Mice were daily administered with PRE-084 (0.25 mg/kg) from 8 to 16 weeks of age. Functional outcome was assessed by electrophysiological tests and computerized analysis of locomotion. Histological, immunohistochemical analyses and Western blot of the spinal cord were performed. PRE-084 administration from 8 weeks of age improved the function of MNs, which was manifested by maintenance of the amplitude of muscle action potentials and locomotor behavior, and preserved neuromuscular connections and MNs in the spinal cord. Moreover, it extended survival in both

female and male mice by more than 15 %. Delayed administration of PRE-084 from 12 weeks of age also significantly improved functional outcome and preservation of the MNs. There was an induction of protein kinase C-specific phosphorylation of the NR1 subunit of the N-methyl-D-aspartate (NMDA) receptor in SOD1^{G93A} animals, and a reduction of the microglial reactivity compared with untreated mice. PRE-084 exerts a dual therapeutic contribution, modulating NMDA Ca²⁺ influx to protect MNs, and the microglial reactivity to ameliorate the MN environment. In conclusion, sigma-1R agonists, such as PRE-084, may be promising candidates for a therapeutical strategy of ALS.

Keywords Motoneuron disease · Amyotrophic lateral sclerosis · Sigma-1 receptor · PRE-084 · SOD1^{G93A} mice

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disorder characterized by progressive muscle atrophy and paralysis due to death of upper and lower MNs [1]. The majority ALS cases are sporadic with unknown etiology, whereas 10 % of cases correspond to inherited forms of ALS. Among these cases, mutations in the gene encoded for the enzyme Cu/Zn superoxide dismutase 1 (SOD1) are observed in approximately 20 % [2]. Based on these genetic mutations, transgenic animal models have been developed for the study of ALS. The most widely used model is a transgenic mouse with a glycine-to-alanine conversion at the 93rd codon of the SOD1 gene in high copy number (SOD1^{G93A}) [3]. The phenotype of this mice recapitulates several clinical and histopathological features of both familial and sporadic forms of the human disease [3]. Moreover, it has been recently reported that alterations of the SOD1 protein are also related to sporadic ALS cases [4], increasing the interest in the study of transgenic animals.

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Several studies using ALS animal models have hypothesized that ALS pathogenesis might be related to glutamate excitotoxicity, oxidative stress, protein misfolding, mitochondrial defects, impaired axonal transport, and inflammation, with all of these factors contributing to MN death [5].

The sigma-1R is a transmembrane protein found in the endoplasmic reticulum [6, 7], which is highly expressed in MNs and other cells in the spinal cord [6, 8–10]. This receptor has the ability to translocate from the endoplasmic reticulum to the plasma membrane and mitochondria-associated membranes [7, 11, 12]. In the nervous system, sigma-1R mediates regulation of several processes, such as neuritogenesis, K⁺ channels, and N-methyl-D-aspartate (NMDA) receptors activity, Ca²⁺ homeostasis, and microglial activity. Furthermore, it is related to some central nervous system pathologies, including depression, schizophrenia, drug addiction, and Alzheimer disease [13–16]. Several studies have demonstrated potent therapeutic actions of sigma-1R agonists, reducing glutamate-mediated cell death [17, 18] or modulating the inflammatory reaction after stroke in rats [19, 20]. Luty et al. [21] and Al-Saif et al. [22] recently reported a novel mutation of sigma-1R in patients affected by ALS, suggesting that pharmacological interventions targeting this receptor could be a good therapeutic approach.

We have previously reported the role of the selective sigma-1R agonist 2-(4-morpholin-1-yl)-1-phenylcyclohexanecarboxylate (PRE-084) in motoneuron death. We demonstrated that PRE-084 administration promotes neuroprotection and neurite elongation through protein kinase C (PKC) on MNs in an *in vitro* organotypic model of excitotoxic lesion [18]. Moreover, administration of PRE-084 prevented spinal MNs death after spinal root avulsion in rats [10]. Therefore, the aim of this study was to assess the potential therapeutic effect of the sigma-1R agonist in the SOD1 mouse model of ALS.

Material and Methods

Transgenic Mice and Drug Administration

Transgenic mice with the G93A human SOD1 mutation (B6SJL-Tg[SOD1-G93A]1Gur) were obtained from the Jackson Laboratory (Bar Harbor, ME), and maintained at the Animal Service of the Universidad de Zaragoza. Hemizygote B6SJL SOD1^{G93A} males were obtained by crossing with B6SJL females from the CBATEG (Barcelona, Spain). The offspring was identified by polymerase chain reaction (PCR) amplification of DNA extracted from the tail tissue. All experimental procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

Animals were evaluated at 8 weeks (prior to drug administration) by electrophysiological tests to obtain baseline values, and as a diagnostic test to distribute them between

the experimental groups. PRE-084 and BD-1036 (Tocris Bioscience, Ellisville, MO) were used as sigma-1R agonist and antagonist, respectively. Both drugs were dissolved in saline and administered daily by intraperitoneal injections at 0.25 mg/kg. The experimental groups included in the study are summarized in Table 1.

Nerve Conduction Tests

Motor nerve conduction tests were performed at 8 weeks of age and then every 2 weeks until 16 weeks in all the animals used in the study (Table 1). The sciatic nerve was percutaneously stimulated by means of single pulses of 0.02 ms duration (grass S88) delivered through a pair of needle electrodes placed at the sciatic notch. The compound muscle action potential (CMAP) (M wave) and the reflex H wave were recorded from the tibialis anterior (TA) and the plantar (interossei) muscles with micro-needle electrodes [23, 24]. All potentials were amplified and displayed on a digital oscilloscope (Tektronix 450S; Tektronix, Beaverton OR, USA) at settings appropriate to measure the amplitude from baseline to the maximal negative peak. To ensure reproducibility, the recording needles were placed under the microscope to secure the same placement on all animals guided by anatomical landmarks. During the tests, the mice body temperature was kept constant between 34 and 36°C by means of a thermostated heating pad.

Treadmill Test

The DigiGait system (DigiGait Imaging System, Mouse Specifics, Boston, MA) was used to assess the locomotor performance of the animals at the end-stage of the disease (at 16 weeks of age). All the animals used in the study (Table 1) were tested. The animals were placed on the treadmill belt and their capacity to run with increasing velocity was recorded. Treadmill speeds used were 5, 10, 15, 20, 25, and 30 cm/s, based on previous studies performed in our laboratory [25].

Table 1 Experimental groups included in the study

Gender	n	Treatment onset	Experimental group
Female	9	—	Wild type
Female	19	8 weeks	SOD1 + saline (SOD1c)
Female	19	8 weeks	SOD1 + PRE (PRE)
Female	9	8 weeks	SOD1 + PRE + BD (PRE + BD)
Female	9	12 weeks	SOD1 + PRE (delayed)
Male	9	—	Wild type
Male	19	8 weeks	SOD1 + saline (SOD1c)
Male	19	8 weeks	SOD1 + PRE (PRE)

BD=BD-1036; PRE=PRE-084; SOD1=superoxide dismutase 1 transgenic; SOD1c=superoxide dismutase 1 transgenic control

Retrotracers Injection

Mice of 15 weeks of age (5 of each group) were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Their left hind paw was gently shaved and the skin was disinfected with povidone-iodine. A small incision in the skin was performed over the TA muscle and a 30-gauge needle connected to a Hamilton syringe (Hamilton Co, Reno, NV, USA) was inserted in the proximal region of the TA muscle. Two μ l of 4 % Fluorogold (Fluorochrome LLC, Denver, CO, USA) were injected directly into the muscle. After the injection, the needle was left in place for 30 s and then slowly withdrawn to avoid liquid from coming out of the muscle.

Histology

One week after retrotracer injection (at 16 weeks of age) mice were transcardially perfused with 4 % paraformaldehyde in phosphate-buffered saline (PBS) and the lumbar segment of the spinal cord was removed, postfixed for 24 h, and cryopreserved in 30 % sucrose. Transverse 40- μ m thick sections were serially cut with a cryotome (Leica CM190, Leica Microsystems, Wetzlar, Germany) between L2 and L5 segmental levels. For each segment, each section from a series of 10 was sequentially collected on separate gelatin-coated slides.

One slide of each animal was rehydrated for 1 min and stained for 2 h with an acidified solution of 3.1 mM cresyl violet. Then the slides were washed in distilled water for 1 min, dehydrated and mounted with DPX (Fluka, Buchs, Switzerland). MNs were identified by their localization in the ventral horn of the stained spinal cord sections and counted after strict size and morphological criteria (only MNs with diameters larger than 20 μ m and with polygonal shape and prominent nucleoli were counted) [10]. The number of MNs present in both ventral horns was counted in 4 serial sections of each L4 to L5 segments [10, 23].

To estimate the number of MNs innervating the TA muscle, retrograde-labeled MNs were counted under epifluorescence microscopy from slides 1, 3, 5, 7, and 9. The total number of MNs was calculated according to the formula of Abercrombie [26].

Another series of sections was blocked with PBS-Triton-fetal bovine serum (FBS) and incubated overnight at 4 °C with primary antibodies anti-glial fibrillary acidic protein (1:1000, Dako, Glostrup, Denmark), rabbit anti-ionized calcium binding adaptor molecule 1 (1:1000, Wako, Chemicals, Richmond, VA, USA), anti-sigma-1R (1:500, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti-phospho-NR1 (Ser896) (1:500, Millipore, Billerica, MA, USA), or anti-phospho-NR1 (Ser897) (1:500, Millipore). After washes, sections were incubated for 1 h at room temperature with Cy2- or Cy3-conjugated secondary antibody (1:200; Jackson ImmunoResearch, West Grove, PA, USA), or, in the case of anti-phospho-NR1 antibodies, with biotinylated secondary antibody (1:200, Vector,

Burlingame, CA, USA) and then for 2 h at room temperature with Cy3-conjugated streptavidin (1:1000, Vector). For co-localizations, spinal MNs were labeled with Cy2- or Cy3-conjugated fluorotracer Nissl staining (1:200, Life Technologies, Grand Island, NY, USA). To quantify astroglial and microglial immunoreactivity, microphotographs of the grey matter of the ventral horn were taken at $\times$ 400 and, after defining the threshold for background correction, the integrated density of glial fibrillary acidic protein (GFAP) or ionized calcium binding adaptor molecule 1 (Iba1) labeling was measured using ImageJ software (National Institutes of Health, Bethesda, MA, USA) [27, 28]. The integrated density is the area above the threshold for the mean density minus the background.

Protein Extraction and Western Blot

For protein extraction, a subset of mice (4 of each experimental group) were anesthetized and decapitated at 16 weeks of age. The lumbar spinal cord was removed and divided into quarters to isolate the ventral parts. One of these parts was prepared for protein extraction and homogenized in modified radioimmuno-precipitation assay buffer (50 mM Tris-HCl pH7.5, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.2 % sodium dodecyl sulfate (SDS), 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA)) adding 10 μ l/ml of Protease Inhibitor cocktail (Sigma, St Louis, MO, USA) and 1 mM sodium orthovanadate (phosphatase inhibitor) (Roche, Mannheim, Germany). After clearance, protein concentration was measured by bicinchoninic acid assay (BCA Protein Assay Kit, Pierce, Rockford, IL).

To perform the Western blot, 30 μ g of protein of each sample were loaded in 7.5 % SDS-polyacrylamide gels. The transfer buffer was 25 mM trizma-base, 192 mM glycine, 20 % (v/v) methanol, pH8.4. The membranes were incubated with 5 % bovine serum albumin in PBS plus 0.05 % Tween-20 for 1 h, and then with primary antibodies at 4 °C overnight. The primary antibodies used were: anti-b-actin (Sigma; 1:10000), anti-sigma-1R (Santa Cruz, 1:500), anti-NMDAR1 (Millipore, 1:500), anti-phospho NR1 (S896) (Millipore, 1:500), or anti-phospho NR1 (S897) (Millipore, 1:500). Horseradish peroxidase, coupled with secondary antibody incubation was performed for 90 min at room temperature. The membranes were visualized using an enhanced chemiluminescence method and the images were collected and analyzed with Gene Genome apparatus and Gene Snap and Gene Tools softwares (Syngene, Cambridge, UK), respectively.

Survival

A subset (of 10 mice of each group and gender) was used to assess the effects of treatment on survival of the animals. It was considered that animals reached the endpoint of the disease when they were unable to stand up for 30 s when placed on their side.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Electrophysiological and locomotion test results were statistically analyzed using repeated measurements and 1-way analysis of variance, applying the Bonferroni post-hoc test when necessary. Histological data were analyzed using the Mann-Whitney *U* test. Survival data was analyzed using the Kaplan-Meier test.

Results

Sigma-1R Expression in SOD1 Animals

We studied the sigma-1R expression levels and localization in the ventral spinal cord of mice aged 16 weeks to assess whether MNs could be directly affected by the PRE-084 administration. Western blot analysis revealed that the expression levels of the receptor were not altered during disease progression because there were no differences in the amount of protein between experimental groups (Fig. 1A). Furthermore, immunohistochemistry revealed co-labeling between Nissl staining (labeling MNs) and sigma-1R (Fig. 1B), indicating that this receptor is expressed in the MNs of both wild-type and transgenic animals.

PRE-084 Administration Improves Spinal Motoneuron Function in SOD1 Animals

The analysis of lower MN functional activity is crucial to assess the effect of new treatments, because the loss of

neuromuscular function is 1 of the hallmarks of the disease process in ALS animal models [29–31]. The results showed that PRE-084 administration significantly improved the amplitude of TA and plantar compound muscle action potential (CMAP), both in female and male SOD1^{G93A} mice from 12 weeks of age compared to untreated mice. This effect was sustained until the end of the follow-up (16 weeks of age) when the mean amplitude of the potentials was twice that of the untreated animals. The functional preservation was especially marked in the TA muscle, because at the end of the follow-up its function was 80 % of the baseline values recorded in the pre-treatment 8-weeks test. Coadministration of the sigma-1R antagonist BD-1036 reverted the improvement achieved by the PRE-084 agonist (Fig. 2).

PRE-084 Treatment Improves Locomotor Performance in SOD1 Animals

PRE-084 administration also significantly improved the locomotor performance of SOD1 animals (Kaplan-Meier test; $p < 0.05$). Both male and female mice administered with the sigma-1R agonist were able to run at higher speeds compared to untreated mice. The performance of untreated SOD1 animals began to decline from 10 cm/s and none were able to run at 30 cm/s (the maximum speed tested). However, animals treated with PRE-084 did not show locomotor impairments until 25 cm/s, and approximately half of them were able to maintain locomotor performance at 30 cm/s at 16 weeks of age. Again the beneficial effects were blocked when the sigma-1R antagonist was co-administered (Fig. 3).

Fig. 1 Sigma-1 receptor (sigma-1R) expression and localization in the lumbar spinal cord. (A) Western blot was used to assess the expression levels of the sigma-1R in the ventral part of the lumbar spinal cord. (B) Quantification of the Western blot evidenced no differences between groups ($F_{3,27} = 0.63$; $p = 0.6013$). (C) Confocal images of L4-L5 motoneurons (MNs) labeled with fluorescent Nissl staining (red) and anti-sigma-1R (green) to co-localize the receptor into the MNs. Note that the expression pattern is similar in wild-type (WT) mice untreated (SOD1c) and PRE-084 treated (PRE) SOD1^{G93A}. Scale bar, 20 μ m

Fig. 2 Electrophysiological tests of male and female animals to evaluate the lower motor function by means of the evoked compound muscle action potential (CMAP). Tibialis anterior (TA) and plantar muscles were evaluated from 8–16 weeks of age in wild-type (WT) mice, SOD1 untreated (SOD1c), treated with PRE-084 (PRE), and co-treated with PRE-084 and BD-1036 (PRE + BD). Daily administration

of PRE-084 significantly improved lower motoneuron (MN) function, especially in the case of the TA because its function was almost maintained until the end of the follow-up (TA muscle: $F_{12,208}=5.63$; $p<0.001$ for females; $F_{8,176}=2.27$; $p<0.05$ for males. Plantar muscle: $F_{12,208}=4.71$; $p<0.001$ for females; $F_{8,176}=15.43$; $p<0.001$ for males). Values are mean $\pm$ SEM. * $p<0.05$ vs WT mice; # $p<0.05$ vs SOD1c

PRE-084 Administration Preserves Neuromuscular Connections in SOD1 Animals

To assess whether PRE-084 administration could maintain the connection between MNs and the skeletal muscles, retrotracers were injected in the TA muscle at 15 weeks and the number of stained MNs in the spinal cord counted 1 week later. The results showed that sigma-1R agonist administration significantly preserved the number of MNs innervating the TA muscle (Fig. 4). PRE-084 treated animals showed 40 % higher number of labeled MNs in the spinal cord with respect to SOD1 untreated mice. This beneficial effect was reverted by coadministration of the sigma-1R antagonist. Figure 4A shows representative images of the cord ventral horns of untreated (SOD1c), treated with PRE-084, and co-treated with PRE-084 and BD-1036 SOD1 transgenic mice.

PRE-084 Administration Preserves Motoneurons in the Anterior Horn of the Lumbar Spinal Cord

The survival of spinal MNs was assessed by counting the number of stained MNs soma in the anterior horns of the lumbar spinal cord in mice of 16 weeks of age, focusing in L4 to L5 segments where the TA and the plantar muscles motor nuclei are represented [32]. MN

counts were restricted to the lateral area of the ventral horn lamina IX, because this population innervates the hind-limb muscles. Neurons smaller than 20 μ m in diameter were excluded from counting, even if they could be atrophic MNs because they were unlikely to be functional.

PRE-084 administration reduced the neurodegenerative process in SOD1 mice (Fig. 5). Treated animals showed a 30 % increase in the number of MNs present in the ventral spinal horns, without differences between males and females. Although SOD1 untreated animals had 16.1 ± 1.6 (male) and 20.1 ± 1.1 (female) MNs per section, PRE-084 treated mice had 34.8 ± 0.9 and 33.9 ± 2.1 , respectively. The coadministration of the sigma-1R antagonist blocked the neuroprotective effect achieved by the agonist (Fig 5B). Figure 5A shows representative images of ventral horns of wild-type, untreated control, treated with PRE-084, and co-treated with PRE-084 and BD-1036 SOD1 mice to illustrate the neuroprotective effect.

PRE-084 Administration Reduces Microglial but Not Astroglial Immunoreactivity in the SOD1 Spinal Cord

It has been extensively reported that glial cells contribute to MN degeneration, both in animal models [33–35] and in ALS patients [36]. Thus, we analyzed both

Fig. 3 Locomotor performance of wild-type, SOD1 untreated (SOD1c), treated with PRE-084 and co-treated with PRE-084 and BD-1036 animals at 16 weeks of age. (A) The graphs represent the proportion of animals that were able to run with increasing velocity and evidence the larger proportion of PRE-084 treated animals that were able to run at higher speeds (Kaplan-Meier test; chi-square=17.27, $p < 0.001$ for females; chi-square=14.96, $p < 0.001$ for males). (B) Representative images of SOD1 untreated animals (SOD1c) and treated animals with PRE-084 (PRE) running at 10 cm/s. Note the misplacement of both paws (black dots) in the untreated SOD1 mouse and the reversion of this sign achieved by PRE-084 administration

astroglial (glial fibrillar acidic protein-positive) and microglial (Iba-1-positive) immunoreactivity in the anterior horns of lumbar spinal cord sections to assess possible modifications of glial cell behavior induced by the treatment.

The results showed that PRE-084 administration significantly reduced microglial, but not astroglial immunoreactivity in the ventral horn of SOD1 animals (Figs. 6 and 7), whereas in both male and female untreated SOD1 mice, Iba-1 immunoreactivity was increased by more than 600 % compared to wild-type mice, PRE-084 treatment reduced it to approximately

300 %. This improvement was reverted when the sigma-1R antagonist was coadministered.

PRE-084 Delayed Treatment Also Produces Functional Improvement in SOD1 Animals

Considering the important effect achieved by the sigma-1R agonist administered from 8 weeks of age (i.e., a presymptomatic stage), we studied that if a more delayed treatment (i.e., from the 12th week) could also ameliorate the animals condition. Electrophysiological tests revealed that PRE-084 treatment produced a

Fig. 4 Retrotracer injection performed to assess the proportion of lumbar motoneurons (MNs) that were still connected to the tibialis anterior (TA) muscle in wild-type (WT) animals, SOD1 untreated animals (SOD1c), animals treated with PRE-084 and co-treated with PRE-084 and BD-1036 at 16 weeks of age. (A) Representative image of a Fluorogold-labeled MN in the lumbar spinal cord. Scale bar,

20 μ m. (B) The quantification of the total number of Fluorogold-stained MNs revealed a 40 % preservation of neuromuscular connections in the PRE-084 compared to SOD1c untreated mice ($F_{3,19}=105.4$, $p < 0.001$ for females; $F_{2,14}=86.68$, $p < 0.001$ for males). Values are mean $\pm$ SEM. * $p < 0.05$ vs WT mice; # $p < 0.05$ vs SOD1c

Fig. 5 Motoneurons (MN) survival in SOD1 animals. (A) Representative images of Nissl stained L4 spinal cord sections from wild-type mice, SOD1 untreated (SOD1c), treated with PRE-084 (PRE), and co-treated with PRE-084 and BD-1036 (PRE + BD). Note the evident improvement after PRE-084 treatment. Scale bar, 500 μ m. (B) The

quantification of MNs per sections in female and male mice showed a 30 % significant improvement in MN preservation ($F_{3,19}=95.53$, $p<0.001$ for females; $F_{2,14}=197.9$, $p<0.001$ for males). Values are mean $\pm$ SEM. * $p<0.05$ vs WT mice; # $p<0.05$ vs SOD1c

significant increase of the TA CMAP from 14 weeks, with a maximum effect at the end of the follow-up, when the mean amplitude was 25 % higher than in untreated SOD1 mice (Fig. 8A). The delayed treatment also improved the locomotor performance of the animals (Kaplan-Meier test, $p<0.05$), whereas SOD1 untreated animals performance declined from 10 cm/seconds to all treated mice supported until 15 cm/seconds. At 30 cm/seconds, when SOD1 controls were completely unable to perform the test, 42 % of the treated animals were able to run (Fig. 8B). MN counts revealed that delayed administration of the sigma-1R agonist produced a significant 13 % increase in the number of stained MN in

the anterior horns of spinal cord sections compared to SOD1 untreated mice (Fig. 8C).

Sigma-1R Agonist Significantly Extends Survival of SOD1 Transgenic Mice

The administration of PRE-084 from 8 weeks of age significantly prolonged survival of both female and male SOD1 mice (Kaplan-Meier analysis, $p<0.001$), whereas untreated female and male SOD1 mice lived 124.1 ± 0.96 and 122.6 ± 3.06 days, respectively, and treated animals survived for 140.5 ± 1.77 and 133.1 ± 3.91 days (approximately 15 and 10 % longer) (Fig. 9).

Fig. 6 Analysis of astroglial reactivity. (A) Representative microphotographs of wild-type (WT) mice, SOD1 untreated (SOD1c) and treated with PRE-084 (PRE) mice immunolabeled for astrocytes (glial fibrillar acidic protein). Scale bar, 125 μ m. (B) Quantification of glial fibrillar acidic protein immunoreactivity in female and male mice. There were no differences between treated and untreated animals ($F_{3,19}=38.52$, $p<0.001$ for females; $F_{2,14}=106.1$, $p<0.001$ for males). Values are mean $\pm$ SEM. * $p<0.05$ vs WT; # $p<0.05$ vs SOD1c

Fig. 7 Analysis of microglial reactivity. (A) Representative microphotographs of wild-type (WT) mice, SOD1 untreated (SOD1c) and treated mice with PRE-084 (PRE) immunolabeled for microglial cells (Iba-1). Scale bar, 125 μ m. (B) Quantification of Iba-1 immunoreactivity in female and male mice. Note the marked reduction of the microglial reactivity after the PRE-084 treatment ($F_{3,19}=17.91$, $p<0.001$ for females; $F_{2,14}=85.7$, $p<0.001$ for males). Values are mean $\pm$ SEM. * $p<0.05$ vs WT; # $p<0.05$ vs SOD1c

Sigma-1R Exerts Modulation of NMDA Receptors through the PKC Pathway

Previously reported, *in vitro* studies indicate that the sigma-1R agonist suppresses NMDA currents in rat retinal ganglion

cells [14] and rescues MNs from excitotoxic-induced death mediated by PKC activation [18]. Thus, we assessed if PRE-084 might exert its therapeutic effect *in vivo* through modulation of NMDA receptors via PKC. To assess this hypothesis, we analyzed the phosphorylation state of 2 PKC-specific

Fig. 8 Effect of delayed administration of PRE-084 from 12 weeks of age. (A) Lower motoneuron (MN) function evaluated by the compound muscle action potential (CMAP) of the tibialis anterior muscle. PRE-084 treatment prevents the decline in spinal MN function ($F_{8,136}=6.71$; $p<0.001$). (B) Locomotor performance of the animals at 16 weeks of age. The graph represents the proportion of animals that were able to run with increasing velocity. Note the greater proportion of PRE-084-

treated animals that were able to run at higher speeds (Kaplan-Meier test, chi-square=8.21, $p<0.05$). (C) Quantification of MNs per sections evidenced a 13 % significant improvement in MN preservation ($F_{2,14}=138.7$, $p<0.001$). (D) Representative images of wild-type (WT) mice, SOD1 untreated (SOD1c) and treated with PRE-084 mice from 12 weeks of age (PRE 12w). Values are mean $\pm$ SEM. * $p<0.05$ vs WT; # $p<0.05$ vs SOD1c

Fig. 9 PRE-084 administration significantly extended SOD1 mice survival. Sigma-1 receptor (sigma-1R) agonist treatment prolonged SOD1 animals life-span for (A) 16 days in females (chi-square=165.3, $p<0.001$) and (B) 11 days in males (chi-square=141.2, $p<0.001$)

serines (Ser896 and Ser897) in the NR1 subunits of NMDA receptors. Because there were no differences between males and females in the functional and histological results, data from both genders was pooled. The results showed a significant increase in the phosphorylation of PKC-specific Ser896 and Ser897 of the NMDA-NR1 subunit in mice treated with the sigma-1R agonist, by 4.3- and 5.5-fold with respect to SOD1 untreated animals, respectively. The coadministration of the sigma-1R antagonist reduced the amount of phosphorylated NR1 to control values (Fig. 10A). To assess whether the phosphorylation was affecting the receptors

expressed in MNs, we performed immunohistochemical colocalization of the phospho-NR1 subunits and the MNs in the lumbar spinal cord. Results revealed that both PKC-specific NR1 phosphorylations were exclusively present in MNs of PRE-084-treated SOD1 animals (Fig. 10B).

Discussion

In this study we have analyzed the effect of the sigma-1R agonist, PRE-084, as a treatment for the SOD1^{G93A} mouse

Fig. 10 Protein kinase C (PKC)-dependent phosphorylation in S897 and S897 of NMDA-NR1 subunits in the anterior horn of lumbar spinal cord of SOD1 animals at 16 weeks of age. (A) Western blots to assess the degree of phosphorylation of the NMDA-NR1 subunits in the anterior horn of lumbar spinal cord. PRE-084 increased the PKC-specific phosphorylation of NR1 subunits. (B, C) Quantification of Western blots revealed a 4.3- and 5.5-fold increase in phospho-NR1 subunits after the treatment with PRE-084 for (B) S896 and (C) S897,

respectively ($F_{3,27}=15.91$, $p<0.001$ for females; $F_{3,27}=11.47$, $p<0.001$ for males). Values are mean $\pm$ SEM. * $p<0.05$. (D) Confocal images of L4 to L5 motoneurons (MNs) labeled with fluorescent Nissl staining (green) and anti-phospho-NR1 (red) to co-localize the phospho-NR1 into the MNs. Note that phosphorylation of the NMDA-NR1 subunit is only visible in the MNs of animals treated with PRE-084. Scale bar, 20 μ m

model of ALS. The results indicate that daily administration of PRE-084 from 8 weeks of age significantly improved spinal MN function, manifested by maintenance of the amplitude of muscle action potentials and improved locomotion behavior, and preserved both neuromuscular connections and MNs survival in the spinal cord. Moreover, sigma-1R agonist prolonged survival in female and male SOD1 animals. Delayed administration of PRE-084 starting at 12 weeks of age also significantly improved functional outcome and MN preservation of SOD1^{G93A} animals. Among other possible effects, further analyses revealed that PRE-084-induced PKC-specific phosphorylation of 2 serines (Ser896 and 897) of the NMDA-NR1 subunit in MNs, and a reduction of the microglial reactivity in the spinal cord of SOD1^{G93A}-treated animals compared with SOD1^{G93A}-untreated mice.

We previously reported the beneficial role of PRE-084 in terms of neuroprotection on both *in vitro* and *in vivo* models of MN death. First we demonstrated that PRE-084 promotes MN survival and neurite outgrowth in an organotypic model of excitotoxic insult [18]. We have also shown a potent neuroprotective effect of PRE-084 treatment on spinal MNs after L4 to L5 root avulsion injury in adult rats [10]. Focusing on ALS, it has been recently described a causative relation of mutations in the *SIGMA-R1* gene in human patients developing juvenile ALS and frontotemporal lobar degeneration-ALS pathology [21, 22], suggesting that this receptor may be a good target for the treatment of ALS. First we assessed the expression levels and localization of the sigma-1R in the ventral part of the lumbar spinal cord of SOD1^{G93A} mice. Despite a slight reduction compared to wild-type mice, the results showed that sigma-1R was present in spinal MNs of SOD1^{G93A} animals, even at 16 weeks of age, pointing out that agonist administration can directly affect MNs. To confirm that the effects of the selective agonist PRE-084 were due to modulation of the sigma-1R, a selective antagonist (BD1036) was coadministered in another group of animals. This coadministration blocked all the beneficial effects, demonstrating that the therapeutic action can be attributed to the activation of the sigma-1R.

Abnormal function of spinal MNs, loss of neuromuscular junctions, and subsequent death of the MNs are key elements of the pathology in human patients and animal models of ALS [1, 29, 37]. Recently, we characterized the pattern of lower and upper MN dysfunction electrophysiologically [23] during disease progression in the SOD1^{G93A} mouse model, revealing that this is a reliable test to monitor the animal model, as well as human patients. In the present work, we found that daily sigma-1R agonist administration from 8 weeks of age, a time when signs of paresis are not present, yet but when nerve conduction tests clearly identify motor dysfunction, greatly improves the function of lower MN in both male and female SOD1^{G93A} mice, as evidenced

by the amplitude of the CMAPs, especially in the TA muscle, which functioned at 16 weeks of age and was maintained compared to baseline values. This improved function correlated with the preservation of more than 70 % of spinal MNs, still innervating the TA muscle, twice as many as in the untreated SOD1^{G93A} animals (32 %). Gait abnormalities and progressive paralysis are also 2 main characteristics of ALS in human [1] and animal models [25]. We found a significant improvement in the locomotor capacity of SOD1^{G93A} mice treated with PRE-084 because they were able to run at higher speeds on a treadmill than untreated SOD1^{G93A} mice. This fact demonstrates that sigma-1R agonist administration preserves muscle strength and reduces signs of paralysis. Furthermore, it resulted in a significant prolongation for 16 and 11 days in survival of female and male SOD1^{G93A} mice, respectively. Therefore, sigma-1R modulation produces important functional benefits in terms of spinal MN function, locomotion, and survival in the SOD1^{G93A} mouse model, even comparatively better than silencing the mutant SOD1 expression [38] or other pharmacological treatments previously reported [39], such as riluzole [40].

Once demonstrated that daily PRE-084 administration from 8 weeks of age significantly improves functional performance and preserves MN in the spinal cord, we performed a second study in which PRE-084 was administered from 12 weeks, the beginning of the classical symptomatic phase in SOD1^{G93A} mice, to mimic the time point at which patients might start the treatment. The results showed that delayed administration was also able to improve spinal MN function and locomotion performance, and significantly preserved MN cell bodies in the spinal cord compared to SOD1^{G93A} untreated animals.

Sigma-1R ligands have been shown to promote neuroprotection in several types of insults [15]. *In vitro* studies revealed potent neuroprotective effects of sigma-1R agonists after excitotoxic damage [18], hypoxia-mediated neurotoxicity [41], oxidative stress-induced cell death [17], or glucose deprivation [42]. *In vivo* studies demonstrated that sigma-1R agonists exert therapeutic actions in rats after stroke [19, 20], after selective cholinergic lesions [43], and after spinal root avulsion [10]. The results found in this study also reveal a potent neuroprotective effect of PRE-084 administration on spinal MNs in the neurodegenerative SOD1^{G93A} mice model. At 16 weeks of age, there was a 30 % increase in the number of surviving MNs in the spinal cord of both male and female SOD1 animals treated with PRE-084 with respect to untreated animals. We have previously described the progression of spinal MN degeneration of SOD1^{G93A} mice, showing that the number of surviving MNs per lumbar cord section averaged 37±2 and 33±3 in male and female mice, respectively, at 12 weeks of age, and 16±2 and 20±1 in male and female mice at 16 weeks of age

[23, 44], respectively. In the present study, we found that PRE-084 treated animals had a mean of 35 ± 1 (males) and 34 ± 2 (females) MNs per section at 16 weeks of age. Thus, sigma-1R agonist administration slows down the degenerative process so that treated animals have the same number of MNs per section at 16 weeks than untreated animals at 12 weeks.

It has been extensively described that calcium dysregulation and excitotoxicity are 2 main pathogenic mechanisms of ALS pathology [45, 46]. ALS-vulnerable spinal and brainstem MNs display low endogenous Ca^{2+} buffering capacity, that is 5 to 6 times lower than that found in ALS-resistant MNs (i.e., oculomotor MNs), making them more susceptible to excitotoxic insults [47]. NMDA receptor is considered to be 1 of the key elements in excitotoxicity [46]. It has been reported that sera from ALS patients induces abnormal NMDA receptor activation [48]. Moreover, Sunico et al. [49] showed an excitation/inhibition imbalance in MNs of SOD1^{G93A} mice with an increased density of glutamatergic synapses, which could lead to an enhancement of Ca^{2+} influx into the cells. Thus, NMDA receptor modulation might be a good target to reduce MN damage. Sigma-1R agonists, such as SKF10097 and PRE-084, suppress NMDA currents in rat retinal ganglion cells through a PKC-dependent mechanism, leading to a reduction of calcium influx into the cytoplasm [14]. Furthermore, sigma-1R agonists prevent Ca^{2+} dysregulation and promote neuroprotection in rat cortical neurons by modulating Ca^{2+} influx through NMDA receptors [41], and also protect MNs in organotypic cultures against excitotoxicity [18]. Our novel results show that sigma-1R agonist administration led to increased PKC-specific phosphorylation of NR1 subunits present in spinal MNs, demonstrating a direct effect of the treatment in these cells. These changes in the NMDA receptor might reduce its permeability and, thus, the calcium influx into the MNs, attenuating excitotoxicity. Further experiments should be performed to assess whether PRE-084 modifies the NMDA channel properties in SOD1 MNs.

Neuroinflammation, and particularly the role of microglia is another pathological hallmark of ALS [50]. In resting conditions, SOD1 transgenic microglia shows reduced neuroprotective properties [51]. Moreover, phagocytic microglia adjacent to MNs is present at the very early stages of the disease in the transgenic rat model of ALS [35], suggesting that abnormal microglial function could play an active role in MN degeneration. Our results revealed a reduction of microglial immunoreactivity in the ventral spinal cord of PRE-084-treated SOD1^{G93A} mice compared to untreated SOD1^{G93A} mice. *In vitro* assays performed by Hall et al. [13], in 2009, showed that the activation of sigma-1R suppresses adenosine triphosphate-induced Ca^{2+} influx and attenuates microglial activation. Both findings point out that sigma-

1R could directly modulate microglial activation in SOD1^{G93A} animals acting through a complementary non-neuronal pathway to ameliorate the MNs environment and promote their survival. This effect may add to the reduction of neuroinflammatory response, secondary to the prevention of motoneuron degeneration.

Our results demonstrate for the first time that a sigma-1R agonist has potent beneficial actions in the SOD1^{G93A} model of ALS. We show that PRE-084 may exert, at least, a dual therapeutic contribution, modulating the NMDA Ca^{2+} influx to reduce excitotoxic damage to MNs, and the microglial reactivity to ameliorate the MNs environment. Such a plurifunctional target may provide better translational outcomes than drugs that act only on 1 of the multiple physiopathological mechanisms involved in ALS. These findings have increased importance because sigma-1R alterations have been reported in human patients, opening a novel perspective for the study of the potential role of sigma-1R agonists as therapeutic agents for ALS.

Acknowledgments This work was supported by Red de Terapia Celular (TERCEL) and Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED) funds, the Fondo de Investigación Sanitaria of Spain (grant no. PI1001787); the Ministerio de Ciencia e Innovación of Spain (grant SAF2009-12495), FEDER funds, and Action COST-B30 of the EC. We thank the technical help of Jessica Jaramillo and Marta Morell. RM is recipient of a predoctoral fellowship from the Ministerio de Educación of Spain.

Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

Conflict of interest The authors report no conflicts of interest.

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