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Lack of synergistic effect of resveratrol and sigma-1 receptor agonist (PRE-084) in SOD1^{G93A} ALS mice: overlapping effects or limited therapeutic opportunity?

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Abstract

Background: Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disease characterized by the loss of motoneurons (MNs) in the spinal cord, brainstem and motor cortex, causing progressive paralysis and death. Nowadays, there is no effective therapy and most patients die 2–5 years after diagnosis. Sigma-1R is a transmembrane protein highly expressed in the CNS and specially enriched in MNs. Mutations on the Sigma-1R leading to frontotemporal lobar degeneration-ALS were recently described in human patients. We previously reported the therapeutic role of the selective sigma-1R agonist 2-(4-morpholinyl)-1-phenylcyclohexanecarboxylate (PRE-084) in SOD1^{G93A} ALS mice, that promoted spinal MN preservation and extended animal survival by controlling NMDA receptor calcium influx. Resveratrol (RSV, trans-3,4',5-trihydroxystilbene) is a natural polyphenol with promising neuroprotective effects. We recently found that RSV administration to SOD1^{G93A} mice preserves spinal MN function and increases mice survival. These beneficial effects were associated to activation of Sirtuin 1 (Sirt1) and AMP-activated protein kinase (AMPK) pathways, leading to the modulation of autophagy and an increase of mitochondrial biogenesis. The main goal of this work was to assess the effect of combined RSV and PRE-084 administration in SOD1^{G93A} ALS mice.

Methods: We determined the locomotor performance of the animals by rotarod test and evaluated spinal motoneuron function using electrophysiological tests.

Results: RSV plus PRE-084 treatment from 8 weeks of age significantly improved locomotor performance and spinal MN function, accompanied by a significant reduction of MN degeneration and an extension of mice lifespan. In agreement with our previous findings, there was an induction of PKC-specific phosphorylation of the NMDA-NR1 subunit and an increased expression and activation of Sirt1 and AMPK in the ventral spinal cord of treated SOD1^{G93A} animals.

Conclusions: Although combined PRE and RSV treatment significantly ameliorated SOD1^{G93A} mice, it did not show a synergistic effect compared to RSV-only and PRE-084-only treated groups.

Keywords: Motoneuron disease, Amyotrophic lateral sclerosis, Resveratrol, Sirtuin 1, AMPK, SOD1^{G93A} mice, Sigma-1 receptor, PRE-084, Combined therapy

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Introduction

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disease characterized by the loss of motoneurons (MN) in the spinal cord, brainstem and motor cortex. It clinically manifests by progressive weakness, muscle atrophy and paralysis [1,2]. The majority of ALS cases are sporadic with unknown etiology but 10% of them are inherited forms, linked to genetic mutations. Mutations in the gene encoding for the enzyme Cu/Zn superoxide dismutase 1 (SOD1) are observed in about 20% of the familial cases of ALS [3]. The transgenic mouse that over-expresses the human mutated form of the SOD1 gene with a glycine to alanine conversion at the 93rd codon is the most studied model [4-6] and recapitulates the main features of both sporadic and familial ALS forms [5]. SOD1 protein alterations have also been reported in sporadic ALS patients [7].

The sigma-1 receptor (sigma-1R) is an endoplasmic reticulum (ER) transmembrane protein [8] highly expressed in spinal MNs [9-11], and specially enriched in postsynaptic sites of C-terminals [12]. Sigma-1R participates in several cellular processes including neuritogenesis, ionic channels conductance, calcium homeostasis and microglial activity [13-16]. It has been implicated in diverse neuropathologies, such as depression, schizophrenia and Alzheimer's disease [17]. A novel mutation of the sigma-1R has been recently described in patients affected by juvenile ALS [18]. Sigma-1R agonists have demonstrated to promote protective effects reducing glutamate-mediated cell death [19,20] or modulating inflammatory reaction following stroke in rats [21]. Indeed, we have previously reported the therapeutic role of the selective sigma-1R agonist 2-(4-morpholinomethyl)-1-phenylcyclohexanecarboxylate (PRE-084) in SOD1^{G93A} ALS mice, that promoted spinal MN preservation and extended animal survival by controlling NMDA receptor calcium influx [22].

Resveratrol (RSV, 3,5,4'-trihydroxy-trans-stilbene) is a polyphenol naturally present in grapes and red wine that has been reported to exert neuroprotective effects on neurodegenerative disease models of Alzheimer's disease [23] and Parkinson's disease [24], and in traumatic [25] and ischemic injuries to the CNS [26]. Previous studies showed protective effects after RSV administration on in vitro ALS models [27,28]. We recently found that RSV administration to SOD1^{G93A} mice preserves spinal MN function and increases their survival. These beneficial effects were associated to activation of Sirtuin 1 (Sirt1) and AMP-activated protein kinase (AMPK) pathways, modulation of autophagy and an increase of mitochondrial biogenesis [29-32].

The pathophysiology of ALS is complex, with several processes contributing to MN death, including glutamate excitotoxicity, oxidative stress, protein misfolding, mitochondrial defects, impaired axonal transport and

inflammation [33,34]. In this context, it is likely that combined therapies targeting several pathophysiological mechanisms could lead to stronger effects and better functional outcomes, potentially resulting in successful clinical translation. Thus, the main goal of this study is to assess if the combination of PRE-084 and RSV treatments may have synergistic effects in the SOD1^{G93A} ALS mice.

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, USA), and maintained at the Animal Service of the Universidad de Zaragoza. Hemizygotes B6SJL SOD1^{G93A} males were obtained by crossing with B6SJL females from the CBATEG (Bellaterra, Spain). The offspring was identified by PCR amplification of DNA extracted from the tail tissue. Mice were kept in standard conditions of temperature (22 ± 2°C) and a 12:12 light:dark cycle with access to food and water *ad libitum*. All experimental procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona, where the animal experiments were performed.

Animals were evaluated at 8 weeks (prior to starting treatments) by rotarod and electrophysiological tests to obtain baseline values. Animals were distributed, according to their progenitors, weight and electrophysiological baseline values, in balanced experimental groups. A RSV-enriched diet [23] was given to groups of treated mice from 8 weeks of age. It has been previously demonstrated that RSV does not affect food intake of the animals [35], thereby assuming a normal food intake of 4 g/animal/day, then RSV was given at a daily dose of 160 mg/kg. PRE-084 (Tocris Bioscience, Ellisville, MO), a Sigma-1R agonist, was dissolved in saline and administered daily by single 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 two weeks until 16 weeks in all the animals used in the study. The sciatic nerve was stimulated percutaneously 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) was recorded from the tibialis anterior (TA) and the plantar (interossei) muscles with microneedle electrodes [36,37]. All potentials were amplified and displayed on a digital oscilloscope (Tektronix 450S) at settings appropriate to measure the amplitude from baseline to the maximal

Table 1 Experimental groups included in the study

| Experimental group | Gender | n |
|--------------------|---------|----|
| Wild type | Females | 10 |
| SOD1 untreated | Females | 23 |
| SOD1 + PRE | Females | 18 |
| SOD1 + RSV | Females | 23 |
| SOD1 + PRE + RSV | Females | 21 |
| Wild type | Males | 10 |
| SOD1 untreated | Males | 10 |
| SOD1 + PRE | Males | 17 |
| SOD1 + RSV | Males | 12 |
| SOD1 + PRE + RSV | Males | 10 |

SOD1: SOD1^{G93A} mice; PRE: PRE-084 (0.25 mg/kg i.p.); RSV: resveratrol (160 mg/kg, via diet).

negative peak. To ensure reproducibility, the recording needles were placed under microscope to secure the same placement on all animals guided by anatomical landmarks. During the tests, mouse body temperature was kept constant by means of a thermostated-controlled heating pad.

Locomotion tests and clinical disease onset

The rotarod test was performed to evaluate motor coordination, strength and balance [38,39] in all the animals used in the study (Table 1). Mice were trained three times a week on the rod rotating at 14 rpm, and then tested from 8 to 16 weeks of age, with an arbitrary maximum time of maintenance in the rotating rod of 180 s. Clinical disease onset was defined as the first day when an animal was not able to complete the 180 seconds on the rotating rod.

Survival

The mice were inspected daily until the standard end point or death. It was considered that animals reached the end point of the disease when they were unable to right themselves in 30s when placed on their side.

Histology

At 16 weeks of age 4–5 mice of each group were transcardially perfused with 4% paraformaldehyde in PBS and the lumbar segment of the spinal cord was harvested, post-fixed for 24 h, and cryopreserved in 30% sucrose. Transverse 40 µm thick sections were serially cut with a cryotome (Leica) between L2-L5 segmental levels. For each segment, each section of a series of 10 was collected sequentially on separate gelatin-coated slides or free-floating in Olmos medium.

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). MNs counts were made for neuronal soma present in the lateral part of lamina IX of the ventral horn in the spinal cord sections and following strict size and morphological criteria: only MNs with diameters larger than 20 µm, polygonal shape and prominent nucleoli were counted. The number of MNs present in both ventral horns was counted in four serial sections of each L4 and L5 segments [36,40], where motor nuclei innervating TA and plantar muscles are located [41].

Another series of sections was blocked with PBS-Triton-FBS and incubated overnight at 4°C with rabbit primary antibody against ionized calcium binding adaptor molecule 1 (Iba1, 1:1000, Wako). After several washes, sections were incubated for 1 hour at room temperature with Alexa 594-conjugated secondary antibody (1:200; Life Science). For co-localizations, spinal MNs were labeled with 500/525-Neurotrace fluorescent Nissl staining (1:200, Life Science). To quantify microglial immunoreactivity, microphotographs of the ventral horn grey matter were taken at × 400 and, after defining the threshold for background correction, the integrated density of Iba1 labeling was measured using ImageJ software. The integrated density represents the area above the threshold for the mean density minus the background.

Protein extraction and western blot

For protein extraction, another subsets of mice (4–5 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 quadrants. One of them was prepared for protein extraction and homogenized in modified RIPA buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 100 mM NaCl, 1 mM EDTA) adding 10 µl/ml of Protease Inhibitor cocktail (Sigma) and PhosphoSTOP phosphatase inhibitor cocktail (Roche). After clearance, protein concentration was measured by Lowry assay (Bio-Rad Dc protein assay).

To perform western blots, 20–30 µg of protein of each sample were loaded in SDS-poliacrylamide gels. The transfer buffer was 25 mM trizma-base, 192 mM glycine, 20% (v/v) methanol, pH 8.4. The membranes were blocked with 5% BSA in TBS plus 0.1% Tween-20 for 1 hour, and then incubated with primary antibodies at 4°C overnight. The primary antibodies used were: anti-GAPDH (MAB374, Millipore, 1:20000), anti-Sirt1 (ab50517, Abcam, 1:1000), anti-p53 (#2524, Cell Signaling, 1:500), anti-acetyl p53 (L382) (06–758, Millipore, 1:500), anti-AMPK (#2532, Cell Signaling, 1:1000), anti-pAMPK (#2531, Cell Signaling, 1:1000), anti-NMDAR1 (AB9864, Millipore, 1:500) and anti-phospho NR1 (S896) (ABN88, Millipore, 1:500). Horseradish peroxidase-coupled secondary antibody (1:5000, Vector) incubation was performed for 60 min at room temperature. The membranes were

visualized by enhanced chemiluminescence method and the images were collected and analyzed with a Gene Genome apparatus using Gene Snap and Gene Tools software (Syngene, Cambridge, UK), respectively.

Statistical analysis

Data are expressed as mean \pm SEM. Electrophysiological and locomotion test results were statistically analyzed using repeated measurements and one-way ANOVA, applying Turkey post-hoc test when necessary. Histological data were analyzed using Mann–Whitney U test. Onset and survival data were analyzed using the Mantel-Cox test.

Results

Combined resveratrol and PRE-084 reduces locomotor impairments and delays clinical disease onset in SOD1^{G93A} mice

We assessed locomotor performance with the rotarod test [38,39]. Rotarod performance was significantly higher in both female and male PRE + RSV treated mice than in control SOD1^{G93A} mice (Figure 1). Furthermore, the combined treatment significantly improved motor outcome of female animals with respect to single RSV or PRE-084 mice at 15 and 16 weeks of age. However, in male mice PRE + RSV did not produce additional benefits compared to single

treatments. Regarding clinical disease onset, PRE + RSV combination significantly delayed the first locomotor signs compared to untreated SOD1^{G93A} mice, but it not represented an improvement in comparison to single treated groups (Figure 1).

Combined resveratrol and PRE-084 preserves spinal motoneuron function in SOD1^{G93A} mice

Lower MN dysfunction is one the main clinical signs of ALS pathology both in animal models [36,41] and human patients [1]. We assessed the functional state of spinal MN by evaluating the amplitude of plantar and TA CMAPs. As previously reported [36] there was a progressive decline in CMAP amplitudes in both muscles along disease progression in SOD1^{G93A} untreated mice. The results revealed that PRE + RSV administration significantly preserves spinal MN function compared to SOD1^{G93A} untreated animals, although it did not promote a better outcome than separated RSV and PRE-084 treatments (Figure 2).

Combined resveratrol and PRE-084 extends survival of SOD1^{G93A} mice

RSV and PRE-084 combined administration from 8 weeks of age significantly extended both female (12.9%) and male (8.7%) lifespan compared to SOD1^{G93A} untreated mice

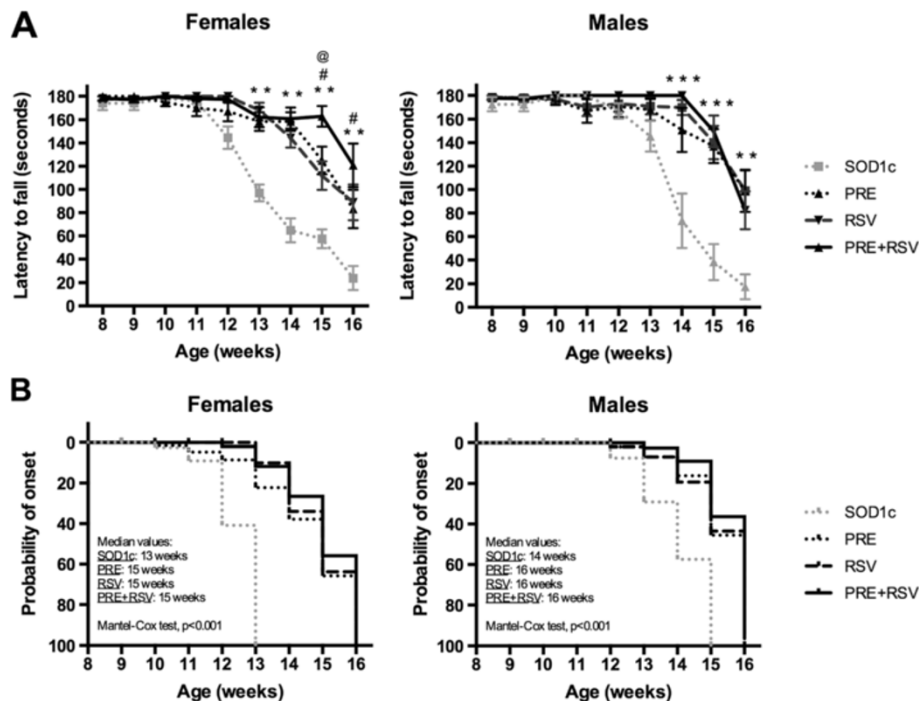


Figure 1 PRE + RSV administration improves locomotor performance and delays disease onset in SOD1^{G93A} mice. **(A)** Rotarod test showed significant improvement in PRE + RSV treated animals compared to untreated littermates. Female but not male SOD1^{G93A} mice treated with PRE + RSV had better performance at advanced stages than RSV and PRE-084 alone treated mice. Values are expressed as mean \pm SEM. **p < 0.01, ***p < 0.001 vs. untreated; #p < 0.05 vs. PRE-084 and @p < 0.05 vs. RSV treated SOD1^{G93A} mice. **(B)** Clinical onset analysis (Mantel-Cox test) revealed that PRE + RSV combination significantly delayed by 2 weeks the signs of disease onset both in female and male SOD1^{G93A} mice.

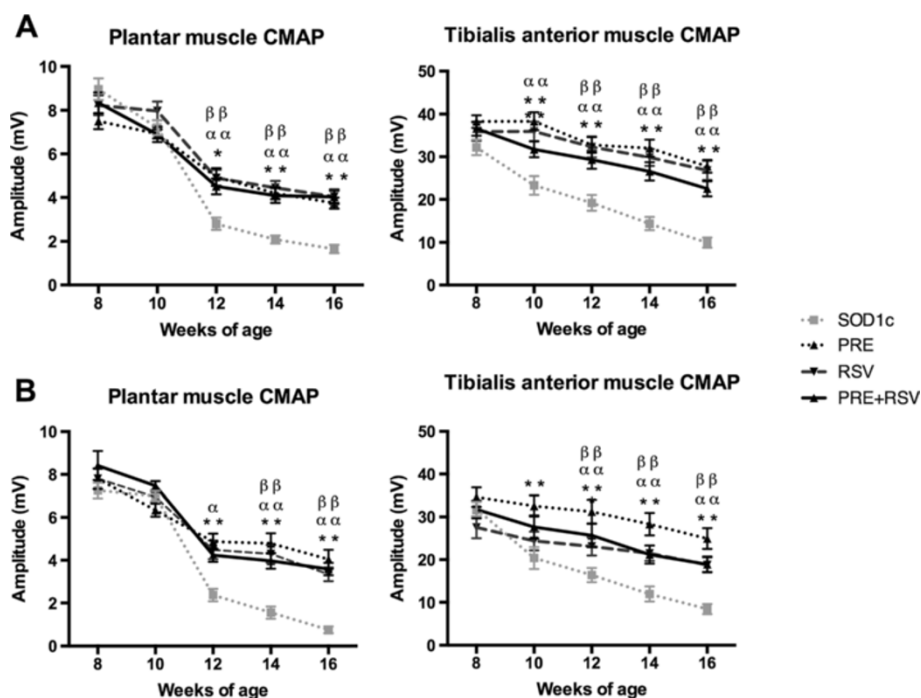


Figure 2 PRE + RSV treatment preserves lower motoneuron function. Electrophysiological tests performed in (A) female and (B) male $SOD1^{G93A}$ mice revealed significant preservation of compound muscle action potentials (CMAP) amplitude in treated groups. Values are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ PRE-084 vs. untreated; α $p < 0.05$, β $p < 0.01$ PRE + RSV vs. untreated; β $p < 0.01$ RSV vs. untreated.

(Mantel-Cox test, $p < 0.001$). The combined treatment also prolonged mice survival with respect to PRE-084 (Mantel-Cox test, $p < 0.01$) but not to RSV only treated $SOD1^{G93A}$ mice (Figure 3).

Combined resveratrol and PRE-084 administration reduces spinal motoneuron degeneration and reduces microglial reactivity in $SOD1^{G93A}$ mice

Spinal MN preservation was assessed by evaluating the number of Nissl stained MN cell bodies in the ventral

horns of lumbar L4-L5 spinal segments in 16 weeks old $SOD1^{G93A}$ mice. Figure 4 shows representative images of Nissl stained anterior horns to illustrate the differences between experimental groups. MN counts of female and male animals were pooled since there were no significant differences between genders. PRE-084 and RSV co-administration from 8 weeks of age significantly increased the number of preserved MNs in $SOD1^{G93A}$ mice. PRE + RSV treated mice had 33.3 ± 1.74 MNs (65.4% vs. wild type) per section whereas $SOD1^{G93A}$ untreated

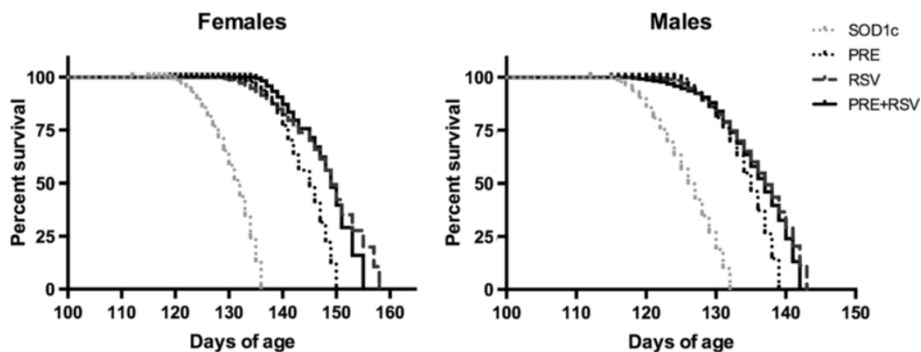


Figure 3 PRE + RSV administration significantly extended $SOD1^{G93A}$ mice survival (Mantel-Cox test, $p < 0.001$). PRE + RSV treatment also increased animals survival compared to PRE-084-only treated group (Mantel-Cox test, $p < 0.05$).

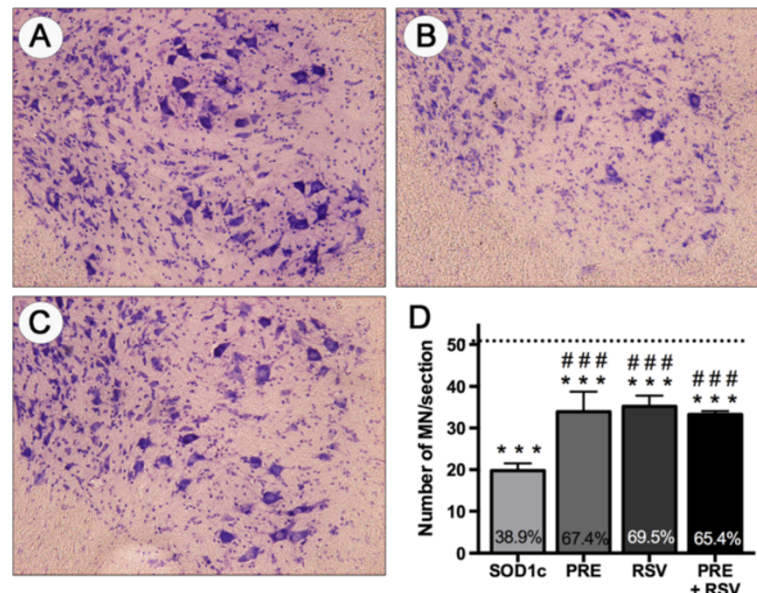


Figure 4 PRE + RSV administration significantly preserves spinal motoneurons from degeneration. Representative images of L4 spinal cord of (A) wild type, (B) SOD1^{G93A} untreated and (C) PRE + RSV SOD1^{G93A} treated mice. Scale bar, 500 μm. (D) L4-L5 spinal cord motoneuron counts revealed significant neuroprotection exerted by PRE + RSV co-administration compared to untreated SOD1^{G93A} but not to RSV-only or PRE-084-only treatments. Dashed line represent the mean number of motoneurons per section of wild type mice. Values are mean ± SEM. ***p < 0.05 vs. wild type, ### p < 0.05 vs. untreated SOD1^{G93A} mice.

animals had 19.8 ± 0.79 (38.9% vs. wild type), almost a two-fold increase in surviving MNs. However, the combinatory treatment did not represent improvement in terms of neuronal preservation compared to single RSV or PRE-084 administered animals (Figure 4).

We assessed the microglia reactivity as a measure of the inflammatory state of the lumbar spinal cord. PRE + RSV administration from 8 weeks of age significantly reduced the microglial immunoreactivity in lamina IX of L4-L5 spinal cord segments. However, similar improvement was observed with single PRE or RSV treatments (Figure 5).

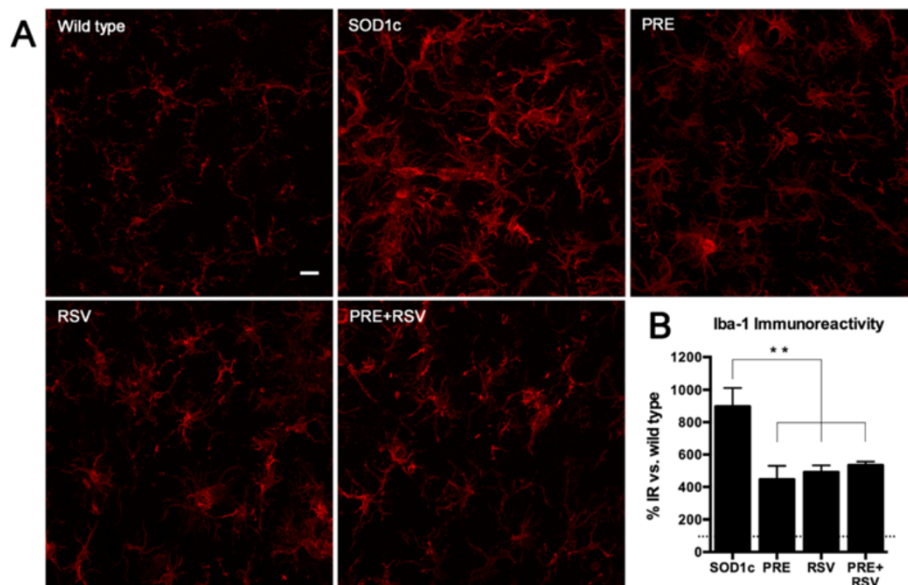


Figure 5 PRE + RSV treatment significantly reduced Iba-1 immunoreactivity in L4-L5 spinal cord lamina IX. (A) Representative microphotographs of L4-L5 spinal cord of wild type, untreated SOD1 (SOD1c), and PRE, RSV and PRE + RSV treated groups. (B) Percentage increase of Iba-1 immunoreactivity compared to wild type values. Values are mean ± SEM. **p < 0.01 vs. SOD1c.

Combined resveratrol and PRE-084 increases Sirt1 and AMPK activation, and promotes specific PKC-dependent phosphorylation of NMDA-NR1 subunits

We have previously shown that RSV treatment induced an increased expression and activation of Sirt1 and AMPK and a consequent modulation of autophagy and mitochondrial biogenesis [32]. On the other hand, PRE-084 administration leads to specific PKC-dependent NMDA-NR1 subunit phosphorylation that may protect MN from degeneration by reducing NMDA calcium currents and thus preventing excitotoxicity [14,22,42]. Therefore, we further analyzed whether RSV and PRE-084 co-administration similarly promotes the activation of the same downstream cellular pathways or there was any interference between drugs.

To assess RSV effects in the combinatory treated $SOD1^{G93A}$ mice we first analyzed Sirt1 levels and

activation in the ventral part of the lumbar spinal cord by western blot analysis. Results revealed a pronounced increase in Sirt1 expression after PRE + RSV co-administration (Figure 6A). To check whether this augmented expression was translated into an enhanced function we analyzed the acetylation state of one the most important Sirt1 targets, p53. We found a significant reduction of acetyl-p53 proportion indicating that Sirt1 was also over-activated (Figure 6B). Secondly, we assessed the activation of AMPK by evaluating active phospho-AMPK fraction. Results showed a marked increase in the pAMPK/AMPK ratio after PRE + RSV co-administration (Figure 6C). These changes were similar to those previously reported after treatment with RSV alone [32].

On the other hand, we evaluated the molecular effects of PRE-084 by analyzing the phosphorylation state of a

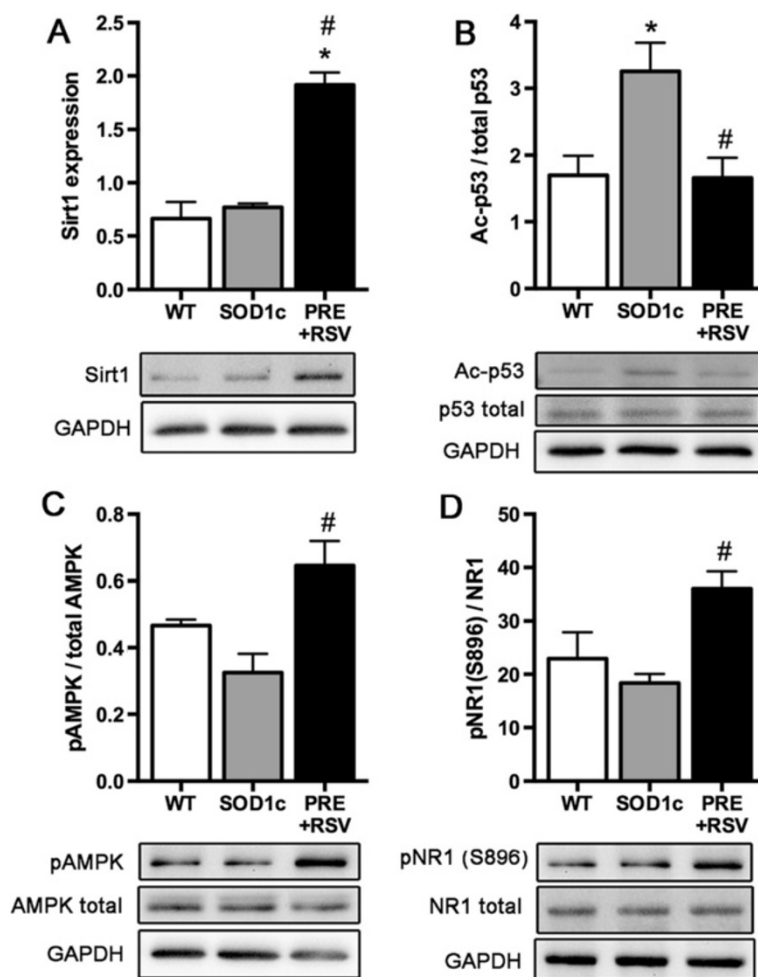


Figure 6 Western blot analysis on the ventral lumbar spinal cord of animals co-administered with PRE + RSV. **(A)** Increased sirtuin 1 expression in $SOD1^{G93A}$ spinal motoneurons after PRE + RSV treatment. **(B)** Evaluation of Sirt1 activity by measuring the acetylation levels of p53 revealed significant deacetylation and thus, increased activity of Sirt1 in $SOD1^{G93A}$ mice treated with PRE + RSV. **(C)** Enhanced AMPK activation after PRE + RSV treatment, evidenced by an increased pAMPK/AMPK ratio. **(D)** Increased Ser896 PKC-specific phosphorylation in NMDA-NR1 subunits of animals treated with PRE + RSV. Values are mean \pm SEM, * $p < 0.05$ vs. wild type, # $p < 0.05$ vs. untreated $SOD1^{G93A}$ mice.

PKC-specific serine (Ser896) in the NMDA-NR1 subunit. Western blot results showed increased Ser896 phosphorylation of the NMDA-NR1 subunit in the animals administered with PRE + RSV (Figure 6D).

Discussion

The main goal of this study was to assess the effect of a novel therapeutic approach combining a Sigma-1R agonist, PRE-084, and RSV in the SOD1^{G93A} mouse model of ALS, since separate administration of the two compounds had resulted in significant improvement of disease progression and survival of these mice [22,32]. Our results indicate that co-administration of PRE-084 and RSV from 8 weeks of age significantly preserved spinal MNs function and reduced MN degeneration together with a reduction of microglial immunoreactivity in the lumbar spinal cord. This effect was accompanied by improvement in the locomotor performance and significant extension of the animals survival. Western blot analyses revealed that, as we previously described, PRE-084 induced PKC-specific phosphorylation of Ser896 of the NMDA-NR1 subunit, whereas RSV increased the expression and activation of Sirt1 and AMPK in the ventral part of the lumbar spinal cord of SOD1^{G93A} mice. Unfortunately, the combinatory therapy did not represent a clear improvement compared to RSV-only or PRE-084-only treated animals.

Mechanisms of neuroprotection and possible overlapping effects

As we previously described, PRE-084 promotes potent neuroprotective effects to MNs both *in vitro* after excitotoxic insults [43], and *in vivo* after spinal root avulsion [11] and in the SOD1^{G93A} mouse model accompanied by a significant extension of animals survival [22]. Calcium dysregulation and excitotoxicity are two pathophysiological mechanisms contributing to ALS pathology [44,45]. In fact, spinal ALS-vulnerable MNs have an endogenous calcium buffering capacity 5–6 times lower than that found in ALS-resistant MNs, increasing their susceptibility to excitotoxic insults [46]. NMDA receptor plays an important role during excitotoxicity [45] and Sigma-1R agonists have been shown to suppress calcium influx to the cells by modulating NMDA receptor through PKC activation. Consistent with our previous observations that PRE-084 administration promotes PKC-specific NMDA-NR1 subunit phosphorylation [22], we have found that PRE + RSV combined treatment also increased Ser896 phosphorylation of the NMDA-NR1 subunit in the ventral part of the spinal cord. Interestingly, increased NMDA-NR1 phosphorylation was only present in the treated group, suggesting that the treatment is activating compensatory protective pathways rather than counteracting a pre-existent pathological event. Although it has been

demonstrated that Sigma-1R physically interacts with NMDA-NR1 subunits [47], it has been also reported that Sigma-1R agonists can modulate ionic flow through calcium, sodium and potassium channels, thus modifying cell excitability properties [14,48,49]. In fact, recent findings by Mavlyutov et al. [50] indicate that the lack of Sigma-1R is detrimental in SOD1^{G93A} mice, probably because it acts by reducing the excitability of spinal MNs. Moreover, Sigma-1R is found associated to ER chaperones (such as BiP) and plays a role in clearance of misfolded proteins by the ERAD response [51,52]. Sigma-1R is enriched in the so-called mitochondrial-associated ER membrane (MAM) and its activation can also modulate mitochondrial metabolism [8,53].

It has been reported that RSV promotes protective effects both in neurodegenerative and traumatic injury models, including Alzheimer's disease and accelerated aging [23,28,54], multiple sclerosis [55,56], Huntington's disease [57,58], Parkinson's disease [24,59], and reducing peripheral axonal degeneration [60] or promoting functional improvements after spinal cord injury [25]. We have recently found that RSV administration significantly delays clinical symptoms onset, improves spinal MNs function and survival, and extends SOD1^{G93A} mice lifespan. We also determined that the therapeutic effect was mediated by the increased expression and activation of both Sirt1 and AMPK, leading to normalization of the autophagic flux and enhanced mitochondrial biogenesis [32]. Although there is some controversy about the exact molecular mechanisms underlying RSV effect, it has been established that Sirt1 or AMPK activation are upstream of pathways that participate in several cellular processes, including inflammation [61-64], autophagy [32] and mitochondrial function [29,54]. Consistent with our previous findings, PRE + RSV treated animals also presented higher expression and activation of both Sirt1 and AMPK compared to SOD1^{G93A} untreated mice. As we previously commented regarding increased NMDA-NR1 phosphorylation, Sirt1 and AMPK overactivation was only present in the treated group, suggesting that the treatment activates protective pathways rather than counteracts a pre-existent pathological condition. This fact may increase the interest of these treatments since the potentiation of endogenous protective mechanisms can be translated to other non-SOD1 ALS situations.

Although the absence of summative effect in the PRE + RSV treated group may be due to an insufficient dose of any of the compounds, a possible overlapping in the pathways activated by both compounds may underlie the lack of synergy. As above mentioned, RSV protective effect is likely related to normalization of the autophagic flux and increased mitochondrial biogenesis [32]. Although PRE-084 main therapeutic effects have been considered associated to modulation of calcium influx and

MNs excitability [14,48,49], it also participates on the response to misfolded protein accumulation [8,52] and the modulation of mitochondrial metabolism [53]. Considering this action, RSV effects on autophagy and mitochondrial biogenesis may mask those of PRE-084 administration thus explaining the lack of summative effect of the combined treatment. Alternatively, both compounds may exert opposite effects on some pathways. For example, it has been reported that Sigma-1R stabilize IRE1 α and increase cell survival through transcriptional activity of X Box binding Protein 1 (XBP1) which in turn regulates genes responsible for protein folding and degradation during the Unfolded Protein Response (UPR) [51]. In contrast, RSV has been shown to suppress the transcriptional activity of XBP1 through Sirt1 [65], therefore promoting an opposite action that could contribute to the lack of synergistic effect of the combinatory treatment assayed.

Another explanation for the lack of summative effect of the combined treatment lies in the pathology state of the animals in the moment we began the drug administration. SOD1^{G93A} mice at 8 weeks of age present 25-30% loss of CMAP amplitude in proximal muscles (TA and gastrocnemius) [36,66], due to early neuromuscular junction retraction and motor axons degeneration [41]. Thus, although MN cell bodies in the anterior spinal cord are intact, up 30% of them are already under a degenerative process that may be irreversible. Since our treatment is focused on the preservation of remaining functional MNs, it is likely that we are acting on the still functioning population of MNs and thus, the maximum effect that can be reached would be limited.

Does the SOD1^{G93A} mouse model present a limited therapeutic capacity?

An alternative explanation for the lack of summative effect after combining PRE-084 and RSV could be that the SOD1^{G93A} mouse model has a limit in terms of MN function and survival that cannot be overpassed by therapeutic interventions. To address this possibility, we made a review of successful preclinical trials using both single and combinatorial treatments in SOD1^{G93A} mice. Additional file 1: Table S1 shows a summary of the therapies performed and the percentage of increased survival compared to untreated mice. It is worth noting that no replication of the positive results was achieved for several of the drugs initially reported to provide efficacy when re-tested using a careful study design [67]. Up to our knowledge and without considering those works showing negative or null results, only 4 of 48 studies reported an extension of survival longer than 25% with just a few showing synergistic effect after combinatory approaches. Although deeper analyses must be performed, such observations may reflect an endogenous limitation for

the therapeutical benefits that can be achieved using the SOD1^{G93A} mouse model.

Conclusions

The main goal of the present work was to assess the therapeutic potential of a combinatory strategy using RSV and PRE-084 in the SOD1^{G93A} mouse model of ALS. Our results revealed that RSV and PRE-084 co-administration significantly improved MN functional preservation and neuroprotection, accompanied by an improvement of the locomotor performance and survival extension. However, this effect was not comparatively better than that achieved by administration of RSV alone.

Additional file

Additional file 1: Table S1. Summary of recent therapeutic trials performed in the SOD1^{G93A} mouse model.

Competing interest

The authors declare that they have not competing interests.

Author's contribution

RM designed the study, performed functional tests and histological studies, collected and analyzed the data, made the figures and prepared the manuscript; JdV performed molecular biology analyses; MM performed the locomotion evaluation; RO bred and genotyped the animals; MP contributed to set the treatment diet and to prepare the manuscript; XN conceived and designed the study, supervised data analysis and prepared the manuscript. All authors read and approved the final manuscript.

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Reference

1. Wijesekera LC, Leigh PN: Amyotrophic lateral sclerosis. *Orphanet J Rare Dis* 2009, **4**:3.
2. Kiernan MC, Vucic S, Cheah BC, Turner MR, Eisen A, Hardiman O, Burrell JR, Zoing MC: Amyotrophic lateral sclerosis. *The Lancet* 2011, **377**:942–55.
3. Rosen DR: Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 1993, **364**:362.
4. McGoldrick P, Joyce PI, Fisher EMC, Greensmith L: Rodent models of amyotrophic lateral sclerosis. *BBA - Molecular Basis of Disease* 1832, 2013:1421–1436.
5. Ripps ME, Huntley GW, Hof PR, Morrison JH, Gordon JW: Transgenic mice expressing an altered murine superoxide dismutase gene provide an

- animal model of amyotrophic lateral sclerosis. *Proc Natl Acad Sci USA* 1995, **92**:689–93.
6. Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, Caliendo J, Hentati A, Kwon YW, Deng HX: **Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation.** *Science* 1994, **264**:1772–5.
 7. Bosco DA, Morfini G, Karabacak NM, Song Y, Gros-Louis F, Pasinelli P, Goolsby H, Fontaine BA, Lemay N, McKenna-Yasek D, Frosch MP, Agar JN, Julien JP, Brady ST, Brown RH: **Wild-type and mutant SOD1 share an aberrant conformation and a common pathogenic pathway in ALS.** *Nat Neurosci* 2010, **13**:1396–403.
 8. Hayashi T, Su T-P: **Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca(2+) signaling and cell survival.** *Cell* 2007, **131**:596–610.
 9. Alonso G, Phan V-L, Guillemain I, Saunier M, Legrand A, Anoaï M, Maurice T: **Immunocytochemical localization of the sigma1 receptor in the adult rat central nervous system.** *Neuroscience* 2000, **97**:155–70.
 10. Palacios G, Muro A, Vela JM, Molina-Holgado E, Guitart X, Ovalle S, Zamanillo D: **Immunohistochemical localization of the sigma1-receptor in oligodendrocytes in the rat central nervous system.** *Brain Res* 2003, **961**:92–9.
 11. Penas C, Pascual-Font A, Mancuso R, Forés J, Casas C, Navarro X: **Sigma receptor agonist 2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate (PRE084) increases GDNF and BiP expression and promotes neuroprotection after root avulsion injury.** *J Neurotrauma* 2011, **28**:831–40.
 12. Mavlyutov TA, Epstein ML, Andersen KA, Ziskind-Conhaim L, Ruoho AE: **The sigma-1 receptor is enriched in postsynaptic sites of C-terminals in mouse motoneurons. An anatomical and behavioral study.** *Neuroscience* 2010, **167**:247–55.
 13. Hall AA, Herrera Y, Ajmo CT Jr, Cuevas J, Pennypacker KR: **Sigma receptors suppress multiple aspects of microglial activation.** *Glia* 2009, **57**:744–54.
 14. Zhang X-J, Liu L-L, Jiang S-X, Zhong Y-M, Yang X-L: **Activation of the ζ receptor 1 suppresses NMDA responses in rat retinal ganglion cells.** *Neuroscience* 2011, **177**:12–22.
 15. Aydar E, Palmer CP, Klyachko VA, Jackson MB: **The sigma receptor as a ligand-regulated auxiliary potassium channel subunit.** *Neuron* 2002, **34**:399–410.
 16. Zhang H, Cuevas J: **σ Receptor activation blocks potassium channels and depresses neuroexcitability in rat intracardiac neurons.** *J Pharmacol Exp Ther* 2005, **313**:1387–96.
 17. Maurice T, Su T-P: **The pharmacology of sigma-1 receptors.** *Pharmacol Ther* 2009, **124**:195–206.
 18. Al-Saif A, Al-Mohanna F, Bohlega S: **A mutation in sigma-1 receptor causes juvenile amyotrophic lateral sclerosis.** *Ann Neurol* 2011, **70**:913–9.
 19. Allahtavakoli M, Jarrott B: **Sigma-1 receptor ligand PRE-084 reduced infarct volume, neurological deficits, pro-inflammatory cytokines and enhanced anti-inflammatory cytokines after embolic stroke in rats.** *Brain Res Bull* 2011, **85**:219–24.
 20. Tuerxun T, Numakawa T, Adachi N, Kumamaru E, Kitazawa H, Kudo M, Kanugi H: **SA4503, a sigma-1 receptor agonist, prevents cultured cortical neurons from oxidative stress-induced cell death via suppression of MAPK pathway activation and glutamate receptor expression.** *Neurosci Lett* 2010, **469**:303–8.
 21. Ajmo CT Jr, Vernon DOL, Collier L, Pennypacker KR, Cuevas J: **Sigma receptor activation reduces infarct size at 24 hours after permanent middle cerebral artery occlusion in rats.** *Curr Neurovasc Res* 2006, **3**:89–98.
 22. Mancuso R, Oliván S, Rando A, Casas C, Osta R, Navarro X: **Sigma-1R Agonist Improves Motor Function and Motoneuron Survival in ALS Mice.** *Neurotherapeutics* 2012, **9**:814–26.
 23. Porquet D, Casadesús G, Bayod S, Vicente A, Canudas AM, Vilaplana J, Pelegrí C, Sanfeliu C, Camins A, Pallás M, del Valle J: **Dietary resveratrol prevents Alzheimer's markers and increases life span in SAMP8.** *Age (Dordr)* 2013, **35**:1851–65.
 24. Wu Y, Li X, Zhu JX, Xie W, Le W, Fan Z, Jankovic J, Pan T: **Resveratrol-activated AMPK/SIRT1/Autophagy in cellular models of parkinson's disease.** *Neurosignals* 2011, **19**:163–74.
 25. Liu C, Shi Z, Fan L, Zhang C, Wang K, Wang B: **Resveratrol improves neuron protection and functional recovery in rat model of spinal cord injury.** *Brain Res* 2011, **1374**:100–9.
 26. Wang L-M, Wang Y-J, Cui M, Luo W-J, Wang X-J, Barber PA, Chen ZY: **A dietary polyphenol resveratrol acts to provide neuroprotection in recurrent stroke models by regulating AMPK and SIRT1 signaling,** thereby reducing energy requirements during ischemia. *Eur J Neurosci* 2013, **37**:1669–81.
 27. Wang J, Zhang Y, Tang L, Zhang N, Fan D: **Protective effects of resveratrol through the up-regulation of SIRT1 expression in the mutant hSOD1-G93A-bearing motor neuron-like cell culture model of amyotrophic lateral sclerosis.** *Neurosci Lett* 2011, **503**:250–5.
 28. Kim D, Nguyen MD, Dobbin MM, Fischer A, Sananbenesi F, Rodgers JT, Delalle I, Baur JA, Sui G, Armour SM: **SIRT1 deacetylase protects against neurodegeneration in models for Alzheimer's disease and amyotrophic lateral sclerosis.** *EMBO J* 2007, **26**:3169–79.
 29. Price NL, Gomes AP, Ling AJY, Duarte FV, Martin-Montalvo A, North BJ, Agarwal B, Ye L, Ramadori G, Teodoro JD, Hubbard BP, Varela AT, Davis JG, Veramini B, Hafner A, Moaddel R, Rolo AP, Coppari R, Palmeira CM, del Cabo R, Baur JA, Sinclair DA: **SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function.** *Cell Metabolism* 2012, **15**:675–90.
 30. Park S-J, Ahmad F, Philp A, Baar K, Williams T, Luo H, Ke H, Rehmann H, Taussing R, Brown AL, Kim MK, Beaven MA, Burgin AB, Manganiello V, Chung JH: **Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases.** *Cell* 2012, **148**:421–33.
 31. Lee IH, Cao L, Mostoslavsky R, Lombard DB, Liu J, Bruns NE, Tsokos M, Alt FW, Finkel T: **A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy.** *Proc Natl Acad Sci USA* 2008, **105**:3374–9.
 32. Mancuso R, del Valle J, Modol L, Martinez A, Granado-Serrano AB, Ramirez-Núñez O, Pallás M, Portero-Otín M, Osta R, Navarro X: **Resveratrol improves motoneuron function and extends survival in SOD1^{G93A} ALS mice.** *Neurotherapeutics* 2014, **11**:419–32.
 33. Ferraiuolo L, Higginbottom A, Heath PR, Barber SC, Greenald D, Kirby J, Shaw PJ: **Dysregulation of astrocyte-motoneuron cross-talk in mutant superoxide dismutase 1-related amyotrophic lateral sclerosis.** *Brain* 2011, **134**:2627–41.
 34. Pasinelli P, Brown RH: **Molecular biology of amyotrophic lateral sclerosis: insights from genetics.** *Nat Rev Neurosci* 2006, **7**:710–23.
 35. Rege SD, Kumar S, Wilson DN, Tamura L, Geetha T, Mathews ST, Huggins KW, Broderick TL, Babu JR: **Resveratrol protects the brain of obese mice from oxidative damage.** *Oxid Med Cell Long* 2013, **4**:1–7.
 36. Mancuso R, Santos-Nogueira E, Osta R, Navarro X: **Electrophysiological analysis of a murine model of motoneuron disease.** *Clin Neurophysiol* 2011, **122**:1660–70.
 37. Navarro X, Udina E: **Methods and protocols in peripheral nerve regeneration experimental research. Electrophysiological evaluation.** *Int Rev Neurobiol* 2009, **87**:105–26.
 38. Brooks SP, Dunnett SB: **Tests to assess motor phenotype in mice: a user's guide.** *Nat Rev Neurosci* 2009, **10**:519–29.
 39. Mancuso R, Oliván S, Osta R, Navarro X: **Evolution of gait abnormalities in SOD1(G93A) transgenic mice.** *Brain Res* 2011, **1406**:65–73.
 40. Penas C, Casas C, Robert I, Forés J, Navarro X: **Cytoskeletal and activity-related changes in spinal motoneurons after root avulsion.** *J Neurotrauma* 2009, **26**:763–79.
 41. Fischer LR, Culver DG, Tennant P, Davis AA, Wang M, Castellano-Sanchez A, Khan J, Polak MA, Glass JD: **Amyotrophic lateral sclerosis is a distal axonopathy: evidence in mice and man.** *Exp Neurol* 2004, **185**:232–40.
 42. McHanwell S, Biscoe TJ: **The localization of motoneurons supplying the hindlimb muscles of the mouse.** *Philos Trans R Soc Lond, B, Biol Sci* 1981, **293**:477–508.
 43. Guzmán-Lenis M-S, Navarro X, Casas C: **Selective sigma receptor agonist 2-(4-morpholinethyl)1-phenylcyclohexanecarboxylate (PRE084) promotes neuroprotection and neurite elongation through protein kinase C (PKC) signaling on motoneurons.** *Neuroscience* 2009, **162**:31–8.
 44. Grosskreutz J, Van Den Bosch L, Keller BU: **Calcium dysregulation in amyotrophic lateral sclerosis.** *Cell Calcium* 2010, **47**:165–74.
 45. Van Den Bosch L, Van Damme P, Bogaert E, Robberecht W: **The role of excitotoxicity in the pathogenesis of amyotrophic lateral sclerosis.** *BBA - Molecular Basis of Disease* 2006, **1762**:1068–82.
 46. Alexianu ME, Ho BK, Mohamed AH, La Bella V, Smith RG, Appel SH: **The role of calcium-binding proteins in selective motoneuron vulnerability in amyotrophic lateral sclerosis.** *Ann Neurol* 1994, **36**:846–58.
 47. Balasuriya D, Stewart AP, Edwardson JM: **The σ -1 receptor interacts directly with GluN1 but not GluN2A in the GluN1/GluN2A NMDA receptor.** *J Neurosci* 2013, **33**:18219–24.
 48. Amer MS, McKeown L, Tumova S, Liu R, Seymour VAL, Wilson LA, Naylor J, Greenhalgh K, Hou B, Majeed Y, Turner P, Sedo A, O'Regan DJ, Li J, Bon RS,

- Porter KE, Beech DJ: Inhibition of endothelial cell Ca²⁺ entry and transient receptor potential channels by Sigma-1 receptor ligands. *British J Pharmacol* 2013, **168**:1445–55.
49. Kourrich S, Hayashi T, Chuang J-Y, Tsai S-Y, Su T-P, Bonci A: Dynamic interaction between sigma-1 receptor and Kv1.2 shapes neuronal and behavioral responses to cocaine. *Cell* 2013, **152**:236–47.
 50. Mavlyutov TA, Epstein ML, Verbny YI, Huerta MS, Zaitoun I, Ziskind-Conhaim L, Ruoho AE: Lack of sigma-1 receptor exacerbates ALS progression in mice. *Neuroscience* 2013, **240**:129–34.
 51. Mori T, Hayashi T, Hayashi E, Su T-P: Sigma-1 receptor chaperone at the ER-mitochondrion interface mediates the mitochondrion-ER-nucleus signaling for cellular survival. *PLoS ONE* 2013, **8**:e76941.
 52. Hayashi T, Hayashi E, Fujimoto M, Sprong H, Su T-P: The lifetime of UDP-galactose:ceramide galactosyltransferase is controlled by a distinct endoplasmic reticulum-associated degradation (ERAD) regulated by sigma-1 receptor chaperones. *J Biol Chem* 2012, **287**:43156–69.
 53. Marriott K-SC, Prasad M, Thapliyal V, Bose HS: σ -1 receptor at the mitochondrial-associated endoplasmic reticulum membrane is responsible for mitochondrial metabolic regulation. *J Pharmacol Exp Ther* 2012, **343**:578–86.
 54. Herskovits AZ, Guarente L: Sirtuin deacetylases in neurodegenerative diseases of aging. *Cell Res* 2013, **23**:746–58.
 55. Fonseca-Kelly Z, Nassrallah M, Uribe J, Khan RS, Dine K, Dutt M, Shindler KS: Resveratrol neuroprotection in a chronic mouse model of multiple sclerosis. *Front Neurol* 2012, **3**:84.
 56. Nimmagadda VK, Bever CT, Vattikunta NR, Talat S, Ahmad V, Nagalla NK, Trisler D, Judge SIV, Royal W, Chandrasekaran K, Russel JW, Makar TP: Overexpression of SIRT1 protein in neurons protects against experimental autoimmune encephalomyelitis through activation of multiple SIRT1 targets. *J Immunol* 2013, **190**:4595–607.
 57. Maher P, Dargusch R, Bodai L, Gerard PE, Purcell JM, Marsh JL: ERK activation by the polyphenols fisetin and resveratrol provides neuroprotection in multiple models of Huntington's disease. *Hum Mol Genet* 2010, **20**:261–70.
 58. Albani D, Polito L, Signorini A, Forloni G: Neuroprotective properties of resveratrol in different neurodegenerative disorders. *BioFactors* 2010, **36**:370–6.
 59. Jin F, Wu Q, Lu Y-F, Gong Q-H, Shi J-S: Neuroprotective effect of resveratrol on 6-OHDA-induced Parkinson's disease in rats. *Eur J Pharmacol* 2008, **600**:78–82.
 60. Araki T, Sasaki Y, Milbrandt J: Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. *Science* 2004, **305**:1010–3.
 61. Zhang F, Liu J, Shi J-S: Anti-inflammatory activities of resveratrol in the brain: role of resveratrol in microglial activation. *Eur J Pharmacol* 2010, **636**:1–7.
 62. Bi XL, Yang JY, Dong YX, Wang JM, Cui YH, Ikeshima T, Zhao YQ, Wu CF: Resveratrol inhibits nitric oxide and TNF-alpha production by lipopolysaccharide-activated microglia. *Int Immunopharmacol* 2005, **5**:185–93.
 63. Candelario-Jalil E, de Oliveira A, Gräf S, Bhatia HS, Hüll M, Muñoz E, Fiebich BL: Resveratrol potently reduces prostaglandin E2 production and free radical formation in lipopolysaccharide-activated primary rat microglia. *J Neuroinflamm* 2007, **4**:25.
 64. Meng X-L, Yang JY, Chen G-L, Wang L-H, Zhang L-J, Wang S, Li J, Wu CF: Effects of resveratrol and its derivatives on lipopolysaccharide-induced microglial activation and their structure-activity relationships. *Chem Biol Interact* 2008, **174**:51–9.
 65. Wang FM, Galson DL, Roodman GD, Ouyang H: Resveratrol triggers the pro-apoptotic endoplasmic reticulum stress response and represses pro-survival XBP1 signaling in human multiple myeloma cells. *Exp Hematol* 2011, **39**:999–1006.
 66. Azzouz M, Leclerc N, Gurney M, Warter JM, Poindron P, Borg J: Progressive motor neuron impairment in an animal model of familial amyotrophic lateral sclerosis. *Muscle Nerve* 1997, **20**:45–51.
 67. Scott S, Kranz JE, Cole J, Lincecum JM, Thompson K, Kelly N, Bostrom A, Theodoss J, Al Nakhala BM, Vieira FG, Ramasubbu J, Heywood JA: Design, power, and interpretation of studies in the standard murine model of ALS. *Amyotroph Lateral Scler* 2008, **9**:4–15.

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