Resveratrol Improves Motoneuron Function and Extends Survival in SOD1^{G93A} ALS Mice

Renzo Mancuso · Jaume del Valle · Laura Modol · Anna Martínez ·
Ana B Granado-Serrano · Omar Ramirez-Núñez · Mercé Pallás ·
Manel Portero-Otín · Rosario Osta · Xavier Navarro

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Abstract Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disease that causes progressive paralysis and death due to degeneration of motoneurons in spinal cord, brainstem and motor cortex. Nowadays, there is no effective therapy and patients die 2–5 years after diagnosis. Resveratrol (trans-3,4',5-trihydroxystilbene) is a natural polyphenol found in grapes, with promising neuroprotective effects since it induces expression and activation of several neuroprotective pathways involving Sirtuin1 and AMPK. The objective of this work was to assess the effect of resveratrol administration on SOD1^{G93A} ALS mice. We determined the onset of symptoms by rotarod test and evaluated upper and lower motoneuron function using electrophysiological tests.

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R. Mancuso · J. del Valle · L. Modol · A. Martínez · X. Navarro
Institute of Neurosciences and Department of Cell Biology,
Physiology and Immunology, Universitat Autònoma de Barcelona,
and Centro de Investigación Biomédica en Red sobre Enfermedades
Neurodegenerativas (CIBERNED), Bellaterra, Spain

A. B. Granado-Serrano · O. Ramirez-Núñez · M. Portero-Otín
Department of Experimental Medicine, Faculty of Medicine,
Universitat de Lleida-IRBLleida, Lleida, Spain

M. Pallás
Unitat de Farmacologia i Farmacognòsia, Facultat de Farmàcia,
Institut de Biomedicina (IBUB), Universitat de Barcelona, and
CIBERNED, Barcelona, Spain

R. Osta
Laboratory of Genetic Biochemistry (LAGENBIO-I3A), Aragon
Institute of Health Sciences, Universidad de Zaragoza, Zaragoza,
Spain

X. Navarro (✉)
Unitat de Fisiologia Mèdica, Facultat de Medicina, Universitat
Autònoma de Barcelona, E-08193 Bellaterra, Spain
e-mail: xavier.navarro@uab.cat

We assessed the survival of the animals and determined the number of spinal motoneurons. Finally, we further investigated resveratrol mechanism of action by means of western blot and immunohistochemical analysis. Resveratrol treatment from 8 weeks of age significantly delayed disease onset and preserved lower and upper motoneuron function in female and male animals. Moreover, resveratrol significantly extended SOD1^{G93A} mice lifespan and promoted survival of spinal motoneurons. Delayed resveratrol administration from 12 weeks of age also improved spinal motoneuron function preservation and survival. Further experiments revealed that resveratrol protective effects were associated with increased expression and activation of Sirtuin 1 and AMPK in the ventral spinal cord. Both mediators promoted normalization of the autophagic flux and, more importantly, increased mitochondrial biogenesis in the SOD1^{G93A} spinal cord. Taken together, our findings suggest that resveratrol may represent a promising therapy for ALS.

Keywords Motoneuron disease · Amyotrophic lateral sclerosis · Resveratrol · Sirtuin 1 · AMPK · SOD1^{G93A} mice

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the death of upper and lower motoneurons (MN) that clinically manifests by progressive muscle atrophy and paralysis [1]. Although the majority of ALS cases are sporadic with unknown etiology, 10 % of them are inherited forms, caused by genetic mutations. Among these, mutations in the gene encoding for the enzyme Cu/Zn superoxide dismutase 1 (SOD1) are observed in about 20 % of the patients [2]. The study of these genetic mutations led to the development of several transgenic animal models of ALS. The most widely used is a transgenic mouse that over-

expresses the human mutated form of the SOD1 gene with a glycine to alanine conversion at the 93rd codon [3]. This model recapitulates most relevant clinical and histopathological features of both familial and sporadic forms of the human disease [3]. It is also of relevance that alterations of the SOD1 protein have been reported in sporadic ALS patients [4], increasing the interest of this murine model. Several mechanisms have been implicated as contributors to MN death in ALS, such as glutamate excitotoxicity, oxidative stress, protein misfolding, mitochondrial defects, impaired axonal transport, and inflammation [5, 6]. Nevertheless, positive experimental results targeting some of these abnormalities have failed to translate into successful human trials [7, 8].

Resveratrol (3,5,4'-trihydroxy-trans-stilbene), a polyphenol found in grapes and red wine, has been reported to exert age-delaying and neuroprotective effects [9, 10]. Despite these well-documented beneficial effects, the mechanisms of action of resveratrol remain controversial. However, it has been recently described that resveratrol can trigger a cascade of intracellular events that converge on Sirtuin 1 (Sirt1), AMP-activated protein kinase (AMPK) and PGC-1 α , as important energy-sensing regulators [11, 12]. Sirt1 is a NAD⁺-dependent deacetylase that has emerged as an important element of the cellular metabolic network. Its activation has been shown to protect against neurodegeneration in several neurodegenerative disorders [13]. In fact, Sirt1 may promote neuroprotection by the modulation of several cellular pathways, such as autophagy [14, 15] and mitochondrial biogenesis [16]. On the other hand, it is also accepted that resveratrol benefits may be mediated through AMPK activation [16, 17]. It has been proposed that resveratrol works primarily by activating AMPK, which then activates Sirt1 indirectly by elevating intracellular levels of its cosubstrate NAD⁺ [18, 19]. Alternatively, resveratrol may first activate Sirt1, leading to AMPK activation via deacetylation and activation of the AMPK kinase LKB1 [20, 21].

Resveratrol administration has been shown to provide beneficial effects on several neurodegenerative disease models, such as Alzheimer's disease [10] and Parkinson's disease [22] and in traumatic [23] and ischemic injuries to the central nervous system [24]. Since previous studies showed that resveratrol administration protects MN on *in vitro* ALS models [25, 26], the main goal of the present work was to assess the potential therapeutic effect of a resveratrol-enriched diet in the SOD1^{G93A} 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, 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 $\pm$ 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 resveratrol administration) 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. All functional and survival assessments were performed by researchers blinded with respect to the group treatment. A resveratrol-enriched diet [10] was given to the groups of treated mice from 8 weeks of age and from 12 weeks of age for the delayed treatment group. Assuming a normal food intake of 4 g/animal/day, resveratrol was given at a daily dose of 160 mg/kg. The control groups received a standard diet, with no differences in manipulations during the study. 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 [27, 28]. For evaluation of the motor central pathways, motor evoked potentials (MEP) were recorded from the TA and plantar muscles in response to transcranial electrical stimulation of the motor cortex by single rectangular pulses of 0.1 ms duration, delivered through needle electrodes inserted subcutaneously, the cathode over the skull overlaying the sensorimotor cortex and the anode at the nose [27, 29]. All potentials were amplified and displayed on a digital oscilloscope (Tektronix 450S) at settings appropriate to measure the amplitude from baseline to the maximal 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, the mice body temperature was kept constant by means of a thermostated heating pad.

Table 1 Experimental groups included in the study.

Experimental group	Gender	n	Treatment onset
Wild type	Females	10	–
SOD1 untreated	Females	20	–
SOD1 + Resveratrol	Females	23	8 weeks
Wild type	Males	10	–
SOD1 untreated	Males	16	–
SOD1 + Resveratrol	Males	22	8 weeks
SOD1 untreated	Females	8	–
SOD1 + Resveratrol	Females	8	12 weeks
SOD1 untreated	Males	8	–
SOD1 + Resveratrol	Males	8	12 weeks

Locomotion Tests

The rotarod test was performed to evaluate motor coordination, strength and balance [30, 31] 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 seconds. Clinical disease onset was defined as the first day when an animal was not able to complete the 180 seconds on the rotating rod.

The DigiGait system (Mouse Specifics, Boston, MA) was used to assess the locomotor performance of the animals at the end stage of the disease (16 weeks of age). The animals were placed over the treadmill belt and their capacity to run with increasing treadmill velocity was recorded. Treadmill speeds used were 5, 10, 15, 20, 25 and 30 cm/s, based on previous studies performed in our laboratory [32, 33].

Survival

For survival assessment, 23 female (10 untreated and 13 resveratrol administered) and 20 male (10 untreated and 10 resveratrol administered) SOD1^{G93A} mice were used. 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 from 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- μ m thick sections were serially cut with a cryotome (Leica) between L2–L5 segmental levels. For each segment, each section of a series of ten 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 were identified by their localization in the ventral horn of the spinal cord sections and counted following strict size and morphological criteria; only MNs with diameters larger than 20 μ m, 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 [27, 34].

Another series of sections was blocked with PBS-Triton-FBS and incubated overnight at 4 °C with primary antibodies anti-glial fibrillary acidic protein (GFAP, 1:1000, Dako), rabbit anti-ionized calcium binding adaptor molecule 1 (Iba1, 1:1000, Wako), or anti-sirtuin 1 (Sirt1, 1:200, Abcam). After several washes, sections were incubated for 1 hour at room temperature with Alexa 488 or Alexa 594-conjugated secondary antibody (1:200; Life Science), or, for anti-Sirt antibody, with biotinylated secondary antibody (1:200, Vector) and then for 1 hour with Alexa 594-conjugated streptavidin (1:1000, Vector). For co-localizations, spinal MNs were labeled with 435/455-Neurotrace fluorescent Nissl staining (1:200, Life Science). To quantify astroglial and microglial immunoreactivity, microphotographs of the ventral horn grey matter were taken at $\times 400$ and, after defining the threshold for background correction, the integrated density of GFAP or Iba1 labeling was measured using ImageJ software [33]. 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 subset of mice (4–5 from 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 μ l/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 μ g of protein of each sample were loaded in SDS-polyacrylamide 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 PBS plus 0.1 % Tween-20 for 1 hour, and then incubated with primary antibodies at 4 °C overnight. The primary antibodies used were: anti-b-actin (Sigma; 1:10000), anti-GAPDH (Millipore, 1:20000), anti-Sirt1 (Abcam, 1:1000), anti-p53 (Abcam, 1:500), anti-acetyl p53 (L382) (Millipore, 1:500), anti-AMPK (Cell Signaling, 1:1000), anti-pAMPK (Cell

Signaling, 1:1000), anti-LC3b (Abcam, 1:200), anti-Beclin 1 (Cell signaling, 1:1000), anti-Fis-1 (ThermoScientific, 1:1000), anti mitofusin 2 (Sigma, 1:1000), anti-porin, (Abcam, 1:1000), anti-complex I subunit 39 kDa (Invitrogen, 1:1000), anti-complex II Fp subunit (Invitrogen, 1:1000), anti-complex III subunit Core 2 (Invitrogen, 1:1000), anti-complex IV subunit I (Invitrogen, 1:1000), and anti-complex V subunit α (Molecular Probes, 1:1000). Horseradish peroxidase–coupled secondary antibody (1:5000, Vector) incubation was performed for 60 min at room temperature. The membranes were visualized using enhanced chemiluminescence method and the images were collected and analyzed with Gene Genome apparatus and Gene Snap and Gene Tolls 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

Resveratrol Treatment Delays the Onset of Symptoms and Improves Locomotion Impairment in SOD^{G93A} Mice

The beginning of symptoms for each animal was considered when it showed the first deficits in locomotor performance in the rotarod test [35]. Results revealed a significant delay of symptoms onset (Mantel-Cox test, $p < 0.05$) in both female and male treated SOD^{G93A} mice of 1 and 2 weeks, respectively, compared to untreated mice (Fig. 1a).

Locomotor performance was assessed with rotarod and DigiGait tests [27, 32]. Rotarod performance was significantly higher in both female and male resveratrol treated mice than in control SOD^{G93A} mice (Fig. 1b). Moreover, we explored the ability of animals to run on a treadmill that induced forced locomotion. The proportion of 16-week-old mice that were able to run at increasing velocities revealed a significant improvement of locomotor performance (Mantel-Cox test, $p < 0.001$) in resveratrol treated mice (Fig. 1c).

Resveratrol Administration Preserves Lower and Upper Motoneuron Function in SOD^{G93A} Mice

Upper and lower MN function impairment is the main feature of ALS pathology both in human patients [1] and animal models [27, 36, 37]. We analyzed the amplitude of plantar and TA CMAP and MEP as a measure of lower and upper MN

functional state, respectively. The CMAP amplitude ranged between 49 and 51 mV for TA muscle and between 6 and 9 mV for plantar muscles in wild type mice, and remained unaltered during all the follow-up. In contrast, there was a progressive decline in CMAP amplitude of SOD^{G93A} mice from 8 to 16 weeks of age in TA and plantar muscles, as previously reported [27]. The results revealed a significant preservation of plantar and TA CMAP amplitude in resveratrol treated mice of both sexes (Fig. 2). Central motor pathways were also protected by the treatment, since the amplitude of MEP was also significantly preserved at the end of the follow-up. Central motor conduction preservation was also evidenced by an increased proportion of animals with recorded MEP responses in resveratrol treated groups at the end stage of the disease (16 weeks of age) (Fig. 2).

Resveratrol Administration Reduces Spinal Motoneuron Degeneration of SOD^{G93A} Mice

The survival of spinal MNs was assessed by evaluating the number of stained MN cell bodies in the anterior horns of the lumbar spinal cord in 16-week-old mice. We focused the analysis on L4–L5 segments where the motor nuclei of TA and plantar muscles are represented [38]. MN counts were restricted to the lateral part of the lamina IX, since this population of MN innervates the hindlimb 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. MN counts of female and male animals were pooled since there were no significant differences between genders. Resveratrol administration significantly reduced MN degeneration in SOD^{G93A} mice. While SOD^{G93A} untreated animals had 18.2 ± 1.3 (35.9 % vs. wild type) MNs per section, resveratrol treated mice had 35.2 ± 1.1 (63.6 % vs. wild type), representing an increase of almost two-fold in surviving MNs (Fig. 3). Figure 3 also shows representative images of ventral horns of wild type, untreated and resveratrol treated SOD^{G93A} mice illustrating the neuroprotective effect.

Resveratrol Administration Reduces Microglial Immunoreactivity in the SOD^{G93A} Mice Spinal Cord

It has been extensively reported that glial cells contribute to MN degeneration both in ALS patients [39] and animal models [40–42]. We evaluated astroglial (GFAP labeled cells) and microglial (Iba-1 labeled cells) immunoreactivity in the anterior horns of lumbar spinal cord sections in order to assess whether resveratrol treatment influenced the response of glial cells. As in the MN counts, female and male animals were pooled due to the lack of differences between them. Results revealed that resveratrol administration significantly reduced microglial but not astroglial immunoreactivity in the anterior horn of SOD^{G93A} mice. Whereas in untreated SOD^{G93A}

Fig. 1 Resveratrol administration delays disease onset and improves locomotor performance in SOD1^{G93A} mice. Continuous lines represent resveratrol SOD1^{G93A} treated animals ($n=23$ females; 22 males) while dashed lines represent SOD1^{G93A} untreated mice ($n=20$ females; 16 males). **a** Disease onset assessed by means of rotarod test revealed a significant delay of symptoms appearance of 1 and 2 weeks for male and female SOD1^{G93A} mice, respectively. **b** Locomotor performance

evaluated with rotarod test showed significant improvement with resveratrol administration. Values are expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. SOD1^{G93A} untreated mice. **c** Locomotor performance evaluated by the Digigait test and expressed as the proportion of 16-week-old SOD1^{G93A} mice that were able to run at increasing treadmill velocities. Results revealed significantly increased locomotor capacity in animals treated with resveratrol ($p < 0.05$, Mantel-Cox test)

mice Iba-1 immunoreactivity was increased over 1152 % compared to wild type mice, resveratrol treatment reduced it to 649 %. GFAP immunoreactivity remained unchanged after the treatment, since untreated and resveratrol treated animals showed similar values of 673 % and 614 % over wild type levels, respectively (Fig. 4).

Resveratrol Treatment Significantly Extends Survival of SOD1^{G93A} Mice

The administration of resveratrol from 8 weeks of age significantly prolonged survival of both female and male SOD1^{G93A} mice (Mantel-Cox test, $p < 0.001$). While untreated female and male SOD1^{G93A} mice lived a median of 134 (mean $\pm$ SEM = 130.7 ± 1.32) and 127 (121.6 ± 2.07) days, treated animals survived for 148 (142 ± 2.76) and 139 (131 ± 2.37) days, respectively (Fig. 5).

Resveratrol Treatment from 12 Weeks of Age also Produces Motor Functional Improvement and Neuroprotection in SOD1^{G93A} Mice

Considering the important effect achieved by resveratrol administered from 8 weeks of age, a pre-symptomatic stage of

SOD1^{G93A} mice, we wondered if a delayed treatment from the 12th week, when signs of the disease are clearly present, could also ameliorate the animals' condition. Electrophysiological analysis revealed that resveratrol treatment led to preservation of spinal MN function, evidenced by increased TA and plantar muscle CMAP amplitudes that became statistically significant at 16 weeks of age with respect to values of untreated mice (Fig. 6a,b). This functional effect was accompanied by significant preservation of the number of surviving MNs in L4–L5 spinal cord segments (Fig. 6c), along with a mild reduction of microglial reactivity ($p = 0.15$, Fig. 6d).

Resveratrol Administration Induces Sirtuin1 Expression and Activation

It has been extensively described that, among other effects, resveratrol promotes neuroprotection by increasing Sirt1 activity [9, 43]. To assess whether Sirt1 was overexpressed, we analyzed Sirt1 levels in the lumbar spinal cord by western blot analysis. Results showed a marked increase in Sirt1 expression in resveratrol treated animals (Fig. 7a). Moreover, immunohistochemistry of the ventral spinal cord revealed that Sirt1 expression was localized in the MNs (Fig. 7b). To check the activity state of Sirt1 we evaluated the acetylation degree of

◀ **Fig. 2** Resveratrol treatment preserves upper and lower motoneuron function. **a** Electrophysiological tests performed in female mice ($n=20$ untreated vs. 23 resveratrol treated SOD1^{G93A} mice). **b** Electrophysiological tests performed in male mice ($n=16$ untreated vs. 22 resveratrol treated SOD1^{G93A} mice). Results in both sexes revealed significant preservation of compound muscle action potentials (CMAP) and motor evoked potential (MEP) amplitudes, and an increased proportion of animals maintaining MEPs at 16 weeks of age. Values are mean $\pm$ SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. SOD1^{G93A} untreated mice

one of its most important downstream targets, p53. Indeed, we found a significant reduction in the acetylation of p53, indicating increased Sirt1 activation in the resveratrol treated group (Fig. 7c).

Resveratrol Treatment Restores Autophagic Flux

Increasing evidence suggests that autophagic abnormalities may contribute to ALS physiopathology [44] and resveratrol has been reported as a modulator of autophagia through Sirt1 activation [45]. Thus, we examined the autophagic state of the animals after resveratrol treatment. The results showed normalization of the early markers of autophagy LC3II and Beclin 1 after resveratrol administration (Fig. 8), suggesting that resveratrol treatment normalizes autophagic flux in the SOD1^{G93A} mouse spinal cord.

Fig. 3 Resveratrol administration significantly preserves spinal motoneurons from degeneration. Representative images of L4 spinal cord at $\times 200$ of **(a)** wild type, **(b)** SOD1^{G93A} untreated and **(c)** resveratrol SOD1^{G93A} treated mice ($n=5$ animals per each gender and treatment). Scale bar, 500 μ m. *Inset boxes* show detail of single motoneurons at $\times 1000$. Scale bar, 20 μ m **(d)** L4–L5 spinal cord motoneurons quantification revealed significant neuroprotection exerted by resveratrol administration ($n=5$ per group). Values are mean $\pm$ SEM. * $p<0.05$ vs. wild type, # $p<0.05$ vs. untreated SOD1^{G93A} mice

Resveratrol Restores Mitochondrial Function and Promotes Mitochondrial Biogenesis through AMPK Pathway

Recent evidence has demonstrated that resveratrol promotes mitochondrial biogenesis through the activation of AMPK [16]. Thus, we first assessed the expression and activation of AMPK by analyzing the active phospho-AMPK and total AMPK forms. Results revealed a significant increase of the pAMPK/AMPK ratio ($p<0.01$) in the ventral part of the lumbar spinal cord in SOD1^{G93A} mice treated with resveratrol (Fig. 9c). Next, we analyzed whether resveratrol treatment led to changes of mitochondrial behavior. We found that SOD1^{G93A} untreated mice had an increased expression of the respiratory chain mitochondrial complexes compared to wild-type mice. In contrast, resveratrol treatment reduced the expression of these mitochondrial complexes, with levels similar to wild type mice (Fig. 9a, b). Finally, we evaluated mitochondrial fission and fusion in the ventral spinal cord. Results revealed a significant increase of Fis-1 expression, a marker of mitochondrial fission without alterations of mitofusin 2, an indicator of mitochondrial fusion. Taken together, these results suggest that the activation of AMPK by resveratrol likely leads to a switch of the mitochondrial response to energetic stress from the upregulation of respiratory chain complexes to a more adaptive increase of mitochondrial biogenesis.

Fig. 4 Resveratrol treatment reduces microglial reactivity. Representative confocal images of microglial cells (Iba-1, *green*) and motoneurons (fluorionissl staining, *blue*) in (a) untreated SOD1^{G93A} and (b) resveratrol treated SOD1^{G93A} mice ($n=5$ animals per gender and treatment). (c) Quantification of Iba-1 immunoreactivity revealed a significant reduction after resveratrol administration. Representative confocal images of

astroglial cells (GFAP, *green*) and motoneurons (fluorionissl staining, *blue*) in (a) untreated SOD1^{G93A} and (b) resveratrol treated SOD1^{G93A} mice. (c) Quantification of GFAP immunoreactivity did not show differences between groups. Scale bars, 20 μ m. * $p<0.05$ vs. wild type, # $p<0.05$ vs. untreated SOD1^{G93A} mice. IR immunoreactivity

Discussion

The physiopathological complexity that characterizes ALS [5, 6] is one of the most limiting factors for the development of successful therapies. Several works have been conducted targeting one or more of the physiopathological mechanisms linked to the disease, but even if they achieved positive experimental results they have failed to translate into successful human therapies [7, 8]. It is conceivable that successful treatments should not only fight pathogenic elements of the disease but also promote cellular neuroprotective pathways providing surviving tools to the MNs. The main goal of the present study was to assess the potential therapeutic effect of resveratrol in the SOD1^{G93A} model of ALS, since it has been reported to produce beneficial neuroprotective effects on other neuropathologies through the modulation of several cellular pathways [9]. Our results reveal that resveratrol significantly delayed disease symptoms onset by 1–2 weeks and importantly improved the locomotor performance of the SOD1^{G93A} animals. Moreover, electrophysiological tests showed maintenance of spinal MN function and, by the first time, a significant improvement of upper MN function. These effects were accompanied by an increased preservation of lower MNs cell bodies in the lumbar spinal cord. Resveratrol administration was also able to significantly extend both female and male

SOD1^{G93A} lifespan. Furthermore, delayed resveratrol administration from the beginning of evident locomotor impairments (12 weeks of age) still produced a significant preservation of spinal MN function and cell bodies in the spinal cord. Further experiments revealed that these therapeutic effects were mediated by the increased expression and activation of Sirt1 and AMPK in the spinal cord, both well-known effectors of resveratrol. Moreover, we observed significant restoration of the autophagic flux normal values and, more importantly, increased mitochondrial biogenesis in the spinal cord of resveratrol treated animals.

It has been previously shown that resveratrol and its downstream effectors Sirt1 and AMPK promote protective effects both in neurodegenerative and traumatic injury models. Indeed, potent therapeutic effects of resveratrol administration were reported in animal models of Alzheimer's disease and accelerated ageing [10, 26, 46], multiple sclerosis [47, 48], Huntington's disease [9, 49], Parkinson's disease [22, 50], and even reducing peripheral axonal degeneration [51] or promoting functional recovery after traumatic spinal cord injury [23]. In this sense, resveratrol has been also reported to exert neuroprotection on *in vitro* models of ALS. Thus, Kim et al. [26] showed that resveratrol reduces cell death on primary cultures of neurons overexpressing the SOD1^{G93A} mutant protein, and Yáñez et al. [52] demonstrated that this

Fig. 5 Resveratrol administration significantly extended SOD1^{G93A} mice survival. Resveratrol treatment prolonged SOD1^{G93A} animals median and maximum life span in (a) female ($n=13$ treated, 10 untreated) and (b) male ($n=11$ treated, 10 untreated) SOD1^{G93A} mice

compound protects from neuronal death triggered by CSF of ALS patients. There is some controversy about the *in vivo* effect of resveratrol administration in SOD1^{G93A} mice. Markert et al. [53] reported that dietary resveratrol given at 25 mg/kg did not produce functional effects, whereas Han

et al. [54] showed that intraperitoneal administration of resveratrol at 20 mg/kg led to improved functional outcome although less important than found in our study. A likely explanation of such discrepancies would be the differences in the dose and the route of administration. Resveratrol

Fig. 6 (a–b) Delayed resveratrol administration from 12 weeks of age significantly preserved lower motoneuron function. Compound muscle action potential (CMAP) amplitude of plantar and tibialis anterior muscles in female and male SOD1^{G93A} mice ($n=8$ treated and 8 untreated for each sex). Resveratrol administration from 12 weeks of age (c) significantly protects spinal motoneurons in L4–L5 spinal cord segments and (d) slightly reduces microglial reactivity ($p=0.15$). Values are mean $\pm$ SEM. * $p < 0.05$ vs. wild type, # $p < 0.05$ vs. corresponding untreated SOD1^{G93A} mice

Fig. 7 Increased sirtuin 1 (Sirt1) expression and activation in SOD1^{G93A} spinal motoneurons after resveratrol treatment. **a** Sirt1 expression was significantly increased in the ventral part of the lumbar spinal cord of resveratrol treated animals. **b** Confocal microphotographs of L4 spinal cord confirmed that this expression was localized inside spinal motoneurons. Scale bar, 20 μ m. *WT* wild type, *SOD1c* untreated SOD1^{G93A} mice,

Resv resveratrol treated mice, *NC* negative control without Sirtuin 1 primary antibody or Fluoromissl staining. **c** Evaluation of Sirt1 activity by measuring the acetylation levels of p53 revealed significant deacetylation and thus, an increased activity of Sirt1 in SOD1^{G93A} mice treated with resveratrol. * $p < 0.05$ vs. wild type, # $p < 0.05$ vs. untreated SOD1^{G93A} mice

pharmacokinetics have been extensively studied, revealing that enteric absorption is up to 80 % after oral administration, while up to 98 % of the compound is excreted 7–15 h after administration [55]. Accordingly, resveratrol treatment should be performed either dietary at high doses or intraperitoneally but with more frequent injections than made in the above-mentioned studies.

Although many studies have been conducted to elucidate the mechanisms of action of resveratrol, they still remain controversial. It is known that resveratrol acts through some specific effector molecules, such as Sirt1 or AMPK [11, 12]. Sirt1 is a NAD⁺-dependent deacetylase that plays an important role regulating cell metabolism and has recently been postulated as a neuroprotective element in several neurodegenerative disorders [13]. Sirt1 induces protective effects since it is upstream of multiple effectors that participate in several cell processes, such as inflammation [43], autophagy

[45] and mitochondrial function [16, 46], all of them related to ALS physiopathology [5, 56]. In the present work, we found that Sirt1 was overexpressed in spinal MNs of SOD1^{G93A} mice after resveratrol administration. Then, we assessed Sirt1 activity by evaluating the deacetylation degree of one of the most important Sirt1 substrates, p53. Indeed, we found significantly reduced acetylation of p53, evidencing an increased activity of Sirt1.

Autophagy serves multiple physiological functions, such as protein degradation, organelle turnover and response to stress. Dysregulation of autophagy has been reported to contribute to ALS pathology both in human patients and animal models [44, 57]. Li et al. [58] reported a progressive increase of the relative amount of LC3b II from 90 to 140 days of age in SOD1^{G93A} mice, accompanied by accumulation of autophagic vesicles. These findings could be the result of autophagic flux impairment. Since it has been hypothesized that Sirt1

Fig. 8 Recovery of normal autophagic flux after resveratrol treatment. **a** LC3b2 / LC3b1 ratio. **b** Beclin 1 expression were normalized by resveratrol administration to SOD1^{G93A} mice. * $p < 0.05$ vs. wild type, # $p < 0.05$ vs. untreated SOD1^{G93A} mice

Fig. 9 Resveratrol administration normalized mitochondrial function and increased mitochondrial biogenesis. **a** Representative western blot of the five different mitochondrial respiratory chain complexes. **b** Quantification revealed significant increase of all complexes expression in control SOD1^{G93A} mice, whereas in resveratrol administered SOD1^{G93A} mice the levels were close to normal. **c** Enhanced AMPK activation after

resveratrol treatment, evidenced by an increased pAMPK/AMPK ratio. **d** Increased mitochondrial biogenesis in resveratrol administered SOD1^{G93A} animals. **e** Mitochondrial fusion was not altered by the treatment. Values are mean±SEM. * $p < 0.05$, *** $p < 0.001$ vs. wild type; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. untreated SOD1^{G93A} mice

plays its protective role through the modulation of autophagy [45, 59], we evaluated the expression of two of the most typical markers of autophagy, LC3 and Beclin-1. Our results revealed that both Beclin-1 and the LC3bII/LC3bI ratio were significantly increased in SOD1^{G93A} mice compared to wild type littermates, evidencing impairment of the autophagic flux. In contrast, after resveratrol administration, both markers were normalized to wild type values. Though the restoration of the autophagic flux may be a direct effect downstream of resveratrol-induced Sirt1 activation, it is also possible that it

could be an indirect consequence derived from the general improvement of the mice condition and, thus, of restoration of MN homeostasis.

On the other hand, recent evidence suggests that the resveratrol-induced Sirt1 activation works through AMPK activation [18]. According to this view, Park et al. [12] recently described that resveratrol promotes AMPK phosphorylation through the inhibition of cAMP phosphodiesterase, thus increasing the amount of cAMP. AMPK then activates Sirt1 indirectly by elevating intracellular levels of its cosubstrate,

NAD+[18, 19]. Alternatively, other authors proposed that resveratrol may first activate Sirt1, leading to AMPK activation via deacetylation and activation of the AMPK kinase LKB1 [20, 21]. In any case, resveratrol administration leads to direct or indirect activation of AMPK, as evidenced in our results by the increased pAMPK/totalAMPK ratio in SOD1^{G93A} treated animals.

Numerous studies have highlighted the common role of mitochondria in the pathogenesis of neurodegenerative diseases [60]. Although there is no consensus about the exact role of mitochondrial abnormalities [61], it is accepted that mitochondrial dysfunction is an important hallmark of ALS pathogenesis [5, 62, 63]. Several authors have shown deficits in mitochondrial function in the spinal cord and muscles of both human patients [64] and animal models of ALS [65–67]. Resveratrol improves mitochondrial function and induces biogenesis, although there is some controversy about whether this effect is mediated by AMPK activation [18] or by Sirt1 [16, 68]. Since both Sirt1 and AMPK were found over-activated in SOD1^{G93A} animals treated with resveratrol, we performed a detailed analysis on mitochondria. Contrary to what was expected, we found a significant increase of all the respiratory chain complexes in SOD1^{G93A} mice compared to wild-type littermates, but complete normalization following resveratrol treatment. Then, we found an increased expression of the fission protein Fis-1 in resveratrol treated SOD1^{G93A} mice, evidencing an active process of mitochondrial biogenesis. Our hypothesis is that resveratrol may promote a switch of mitochondrial response to cellular stress from the overexpression of respiratory chain complexes and the consequent increased production of harmful oxidative mediators, to a more adaptive response by increasing mitochondrial biogenesis. It has also been reported that resveratrol administration promotes mitochondrial biogenesis in skeletal muscles [12, 16] through AMPK activation. Further studies are needed to assess whether resveratrol benefits in SOD1^{G93A} mice could be partially explained by its effect on skeletal muscles.

Neuroinflammation is another common pathological hallmark of neurodegenerative disorders [69] and its modulation has been proposed as a potential therapeutic target [70]. In ALS, the inflammatory response is characterized by the activation and proliferation of microglia, and the infiltration of T cells into the spinal cord. Although it is still unknown whether it is a cause or a consequence of the MN degeneration, some evidence suggested that its manipulation could ameliorate the disease progression. Resveratrol has been found to modulate neuroinflammation in both *in vivo* and *in vitro* models, specifically targeting activated microglial cells [71]. Actually, resveratrol suppresses the activation of the NF- κ B pathway in LPS-activated microglia by reducing the phosphorylation and consequent degradation of its inhibitor, I κ B [72–75]. Our results demonstrate that microglial, but not astroglial reactivity, was significantly diminished with resveratrol

administration compared to untreated SOD1^{G93A} mice. Although further studies should be performed to assess the exact effect, resveratrol modulates microglia activity, thus contributing to the neuroprotection observed in resveratrol administered SOD1^{G93A} mice.

The results of this work demonstrate that resveratrol exerts potent therapeutic actions in the SOD1^{G93A} model of ALS. Here, we show for the first time a treatment that combines significant preservation of both lower and upper MN function, translating into significantly delayed disease onset and extended animal survival. These effects were associated with an important preservation of MN and a significant reduction of microglial reactivity in the spinal cord. Molecular analyses revealed that the beneficial effects of resveratrol were accompanied by an activation of Sirt1 and AMPK. Such plurifunctional targets that combine the inhibition of detrimental processes and the promotion of neuroprotective pathways may provide better translational outcomes than drugs with more restricted targets. These findings strongly suggest that resveratrol may be a promising therapy for motoneuron diseases.

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