

Original Article

ICA-27243 improves neuromuscular function and preserves motoneurons in the transgenic SOD1^{G93A} miceVera M. Masegosa^{a,b}, Xavier Navarro^{a,b}, Mireia Herrando-Grabulosa^{a,b,*}^a Department of Cell Biology, Physiology and Immunology, Institute of Neuroscience, Universitat Autònoma de Barcelona, Bellaterra, Spain^b Centro de Investigación Biomédica en Red de Enfermedades Degenerativas (CIBERNED), Instituto de Salud Carlos III, Madrid, Spain

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by the death of upper and lower motor neurons (MNs). Excessive neuronal excitability has been implicated in MN degeneration; thus, modulation of hyperexcitability appears as a promising therapeutic strategy. Potassium channels are attractive targets since they can be activated at subthreshold voltages and can regulate neuronal excitability. In this study, we assayed the effects of N-(6-Chloro-pyridin-3-yl)-3,4-difluorobenzamide compound, known as ICA-27243, as a potential treatment for ALS. ICA-27243 is a highly selective Kv7.2/7.3 opener used mainly in epilepsy models. In the *in vitro* model of spinal cord organotypic cultures (SCOCs) exposed to acute excitotoxicity, ICA-27243 prevented MN degeneration at a dose-of 10 μ M. Administration of ICA-27243 to transgenic SOD1^{G93A} ALS mice improved the decline of neuromuscular function, maintained locomotion and coordination in the rotarod, decreased spinal MN death and attenuated glial reactivity. In conclusion, we report here for the first time that ICA-27243 is an effective treatment for ALS, emphasizing the potential of targeting Kv channels to reduce neuronal hyperexcitability.

Introduction

Amyotrophic lateral sclerosis (ALS), the most common motor neuron disease (MND), is characterized by the selective death of upper and lower motor neurons (MNs). Several physiopathological mechanisms have been identified as contributors to MN degeneration in ALS, including mitochondrial dysfunction, oxidative stress, endoplasmic reticulum (ER) stress, excitotoxicity, protein misfolding, and others [1,2]. One of the most relevant mechanisms is neuronal hyperexcitability, a state where the neuron displays an abnormally high level of excitability, leading to decreased threshold for firing action potentials [3]. Different causes have been proposed for this hyperexcitability, including increased Na⁺ conductance and decreased K⁺ conductance [4,5], loss of inhibitory function in GABAergic and glycinergic interneurons [6], and disruptions in K⁺ homeostasis due to perisynaptic astrocyte dysfunction [7]. Hyperexcitability has been found in both ALS patients and ALS animal models [8], and reported to occur at the early stages of the disease, prior to MN loss [9,10]. As the disease progresses, a shift from hyperexcitability to hypoexcitability takes place [9,11]. Cortical hyperexcitability is attributed to a persistent increase in Na⁺ current density and a hypothesized reduction in K⁺ conductance [12]. Therefore, we propose that

activating Kv7.2/7.3 channels in order to enhance K⁺ conductance, and thereby reduce membrane excitability, could be an effective therapy for ALS.

The Kv7 family is a five-member K⁺ channel encoded by KCNQ genes. KCNQ channels are attractive targets to reduce excitability because: i) they are outwards channels that can be activated at subthreshold potential; ii) they stabilize the membrane potential in the presence of depolarizing currents; iii) they can suppress spiking activity and reduce burst-firing; iv) they are present at the axon initial segment, meaning they are crucial in regulating action potential threshold [13–15]. Additionally, the heteromeric form of Kv7.2/7.3 underlies the M-current, a slowly activating and non-inactivating K⁺ current that regulates neuronal excitability by hyperpolarizing the membrane potential and reducing the firing rate of action potentials.

Based on its intrinsic characteristics, activation of the Kv7.2/7.3 channel has been used for the treatment of conditions associated with neuronal hyperexcitability, such as epilepsy, migraine, anxiety and addiction to psychostimulants [13]. Retigabine, a specific activator of Kv7.2/7.3, reduces hyperexcitability, ROS production and MN bursting, and prevents MN loss in rat hypoglossal MN cultures exposed to excitotoxicity [16]. Moreover, retigabine dose-dependently increased survival

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of SOD1^{A4V/+} ALS hiPSC-derived MNs [17], and decreased the firing rate of hiPSC-derived and mouse MNs [18]. Retigabine was also shown to reduce both cortical and spinal excitability in ALS patients [19,20]. However, the FDA issued a warning regarding the risk of retinal damage and potential vision loss associated with the use of retigabine (Reference ID: 3369445; [accessdata.fda.gov](https://www.accessdata.fda.gov)).

Another selective ligand of Kv7.2/7.3 channels is the N-(6-Chloropyridin-3-yl)-3,4-difluorobenzamide compound, also known as ICA-27243. ICA-27243 induced a reversible increase in Kv7.2/3 current amplitude, and shifted the voltage-dependent activation of Kv7.2/7.3 to hyperpolarized potential and suppressed seizure-like activity in CHO and in SH-SY5Y cells in culture [21]. Its anti-seizure efficacy was demonstrated in different rodent models [22]. ICA-27243 and retigabine could also attenuate chronic inflammatory pain [23]. However, ICA-27243 has not been tested in any MND model. In this study, we assayed for the first time the effectiveness of the therapy with the Kv7.2/7.3 channel activator ICA-27243 in *in vitro* and *in vivo* models of MN degeneration.

Methods

Spinal cord organotypic cultures

Spinal cord organotypic cultures (SCOCs) were prepared as previously described [24,25]. Shortly, spinal cords from P7–P8 Sprague-Dawley rats were aseptically collected and placed in ice-cold Gey's Balanced Salt Solution (Cat. #G9779, Sigma-Aldrich) containing glucose (6.4 mg/mL). After removing the meninges, the spinal cords were sliced transversely into 350 µm thick sections using a McIlwain Tissue Chopper (The Mickle Laboratory Engineering Co.). The L4-L5 lumbar sections were then transferred onto Millicell-CM porous membranes (0.4 µm; Millipore, Burlington, MA, USA) and placed in a six-well plate containing 1 mL of incubation medium, consisting of 50 % MEM (Cat. #M5775, Sigma), 25 mM Hepes (Cat. #H4034-25G, Sigma), 25 % heat-inactivated Horse Serum (Cat. #2605088, Gibco), 2 mM glutamine (Cat. #101806, INC Biomedical Inc.), and 25 % Hank's Balanced Salt Solution (HBSS) supplemented with 25.6 mg/mL glucose (Cat. #14175-095, Gibco; pH 7.2). After 7 days *in vitro* (DIV) at 37 °C and 5 % CO₂ for culture stabilization, the medium was changed twice a week until 15 DIV.

At 15 DIV, excitotoxicity was induced in SCOCs by exposing slices to Locke solution (137 mM NaCl, 2.5 mM CaCl₂, 5 mM KCl, 5.6 mM D-glucose, 0.3 mM KH₂PO₄, 4 mM NaHCO₃, 0.3 mM Na₂HPO₄, 0.01 mM glycine and 10 mM HEPES; pH 7.2) containing L-glutamic acid (Cat. #G8415, Sigma-Aldrich) at a final concentration of 50 µM for 30 min. Immediately after, ICA-27243 (N-(6-chloro-pyridin-3-yl)-3,4-difluorobenzamide, synthesized by Grünenthal GmbH) was added at 2.5 µM, 5 µM, 10 µM and 20 µM in DMSO. Locke solution without glutamate was added to the control. Slices were maintained until 20 DIV at 37 °C and 5 % CO₂. Next, slices were fixed with 4 % paraformaldehyde (PFA), and immunostained to assess MN survival, as described below.

MN viability assessment

As previously described [26], slices were blocked with 5 % normal horse serum (Cat. #S0910500, Biowest) and 0.3 % Triton-X-100 in PBS (PBS-TX) and incubated for 48 h with primary anti-neurofilament H non-phosphorylated antibody (SMI-32; 1:250, Cat. #801701, Biolegend). Subsequently, slices were incubated with secondary antibody Alexa Fluor 488 donkey anti-mouse IgG (1:500, Cat. # A21202, Invitrogen), washed and mounted on glass slides. Z-stack fluorescence images were acquired with a Zeiss LSM 510 Meta confocal microscope. MNs were identified by SMI-32 positive immunostaining, that has been proven to label the cholinergic alpha MNs [27], localization in the ventral horn and morphological features (polygonal shape and clear dendrites). Blind counting of all SMI-32⁺ cells meeting these criteria was performed in each stack of the spinal cord hemisection using the Cell Counter tool from

ImageJ software. Each condition was replicated in 3–4 independent cultures.

Animals and treatment

Female transgenic SOD1^{G93A} mice (B6SJL-Tg [SOD1-G93A] 1Gur) and their non-transgenic wild-type (WT) littermates were utilized in the study. Transgenic offspring were identified by PCR amplification of DNA extracted from the tail using the next primer sequences: 1) hSOD1-forward CATCAG CCCTAATCCATCTGA, 2) hSOD1-reverse CGCGAC-TAACAA TCAAAGTGA, 3) mL2-forward CTAGGCCACAGAATTGAA AGATCT and 4) mL2-reverse GTAGGTGGAAATTCTAGC ATCATCC. All animals were maintained under standard conditions and handled in accordance with the guidelines set by the European Union Council (Directive 2010/63/EU). The experimental protocols were approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

To assess the neuroprotective effect of ICA-27243 female mice were distributed in the following experimental groups: WT untreated (n = 7), WT + ICA-27243 10 mg/kg (n = 6), SOD1^{G93A} + vehicle (n = 5), and SOD1^{G93A} + ICA-27243 10 mg/kg (n = 6). ICA-27243 was administered daily intraperitoneally (i.p.) throughout the experiment at 10 mg/kg/day. ICA-27243 was dissolved in 5 % dimethyl sulfoxide (DMSO) + 95 % hydroxypropyl methylcellulose 0.5 % (HPMC) (Cat. #H9262, Sigma), adding 2 % Tween 80 (Cat. #1754, Sigma). The dose was chosen based on previous studies in which this dose was effective in reducing seizure activity [22] and as an anti-maniac treatment [28]. Previous *in vivo* studies demonstrated that ICA-27243 has good biodistribution and reaches the CNS in rodents at high concentration [22].

Electrophysiological tests

Baseline motor nerve conduction tests were performed at 7 weeks of age (prior to drug administration) to balance the groups. Then, tests were carried out at 8, 12, 14 and 16 weeks of age. The sciatic nerve was stimulated using single pulses of 20 µs duration delivered from a Grass S88 stimulator via needle electrodes placed at the sciatic notch. The compound muscle action potential (CMAP) was recorded from the tibialis anterior (TA) and plantar interossei (PL) muscles with microneedle electrodes, with the active electrode placed on the muscle and the reference at the fourth toe [29]. Muscle evoked potentials (MEP) were recorded from TA muscle to assess the central descending pathways by stimulating the motor cortex by single pulses of 100 µs duration delivered through subcutaneous electrodes, with the cathode placed over the skull overlaying the sensorimotor cortex and the anode at the nose [30]. MEP recordings in rodents exhibit two different components, a first component is associated to the brainstem evoked potential and the second related to the cortical evoked potential.

Locomotion tests

Motor coordination and locomotion were assessed using the rotarod test and treadmill test. The rotarod test was conducted by placing mice on a rotating rod at 14 rpm and measuring the longest latency before falling, with an arbitrary cut-off time of 180 s. This test was carried out weekly from 8 to 16 weeks of age and the first week in which mice were unable to walk for the full 180 s was determined as the onset of disease. The treadmill test was carried out at the end of the follow-up. It consists of gradually increasing the speed from 6 rpm to 30 rpm, and recording the maximum velocity at which mice could maintain the treadmill pace.

Histological and immunofluorescence analyses

At 16 weeks of age, all the mice were euthanized by administering an overdose of sodium pentobarbital and transcardially perfused with 4 % paraformaldehyde in PBS. The lumbar segment of the spinal cord and the TA muscle were harvested and cryopreserved in 30 % sucrose

solution in PBS. To assess MN survival, the L4-L5 segment of the spinal cord was serially cut into 20 μm thick transverse sections using a cryostat (Leica) and collected sequentially on 10 slides. Cresyl violet 3.1 mM stain was applied to the slices for 3 h. Then, slides were washed, dehydrated, and mounted with DPX. MNs were identified based on their location in the lateral ventral horn and strict morphological and size criteria, including polygonal shape, prominent nucleoli, and diameter larger than 20 μm .

To assess microglial and astroglial reactivity, other series of lumbar sections were blocked with 10 % normal donkey serum (Cat. #S30, Millipore) with 0.2 mM glycine (Cat. #G8898, Sigma) in PBS-Tx 0.3 %. Then, sections were incubated overnight at 4 °C with primary antibody rabbit anti-ionized calcium-binding adapter molecule 1 (Iba-1; 1:500, Cat. #019-19741, Wako) and rat anti-glial fibrillary acidic protein (GFAP; 1:500, Cat. #13-0300, Invitrogen). Following several washes, the sections were incubated with the appropriate secondary antibody: Cy3 donkey anti-rabbit (1:500; Cat. #AP182C, Millipore) and Alexa Fluor 488 donkey anti-rat (1:500; Cat. #A-21208, Invitrogen). Sections were dehydrated and mounted with DPX (ThermoFisher). To assess glial cell reactivity, images of the ventral horn of slices were captured at 40 $\times$ with a fluorescence microscope (Olympus BX51) under identical conditions. The integrated density was measured using Image J software.

In order to evaluate the innervation of neuromuscular junctions (NMJ), the TA muscle was longitudinally cut into 50 μm thick sections. Sections were blocked with 10 % normal donkey serum in PBS-Tx 0.3 % for 1 h, and then incubated for 48 h at 4 °C with primary antibodies including anti-neurofilament 200 (NF200) (1:1000; Cat. #AB5539, Millipore) and anti-synaptophysin (1:500; Cat. #AB32127, Abcam). Sections were washed and incubated overnight at 4 °C with Alexa Fluor 594 conjugated secondary antibody (1:200; Cat. #A11042 and #A21207, Invitrogen) and Alexa Fluor 488 conjugated alfa-bungarotoxin (1:200; Cat. #B13422, Life Technologies). The sections were mounted in Fluoromount-G medium (SouthernBiotech, USA) and Z-stack fluorescence images were obtained using a Zeiss LSM 510 Meta confocal microscope. The proportion of innervated NMJs was determined by categorizing each endplate as occupied (innervated or partially innervated) or vacant (denervated), with at least 60 endplates analyzed per animal.

Statistical analysis

Data analyses were conducted using GraphPad Prism 9 software. All data are expressed as mean $\pm$ SEM. The electrophysiological and functional measurements were subjected to repeated-measures two-way ANOVA, while histological data were analyzed using one-way ANOVA. Multiple comparisons were performed using the post-hoc Bonferroni test. Differences were considered significant at $*p < 0.05$.

Results

ICA-27243 prevents MN degeneration in SCOCs under excitotoxic conditions

In SCOCs, the number of MNs preserved, assessed as the number of SMI-32⁺ cells in the ventral horn, was significantly reduced with the addition of 50 μM glutamate for 30 min (61 ± 2 % MN preserved) compared to control slices (100 ± 3 % MN preserved) (Fig. 1). We found that the application of 10 μM ICA-27243 significantly prevented MN death, with MN survival rate of 82 % (82 ± 5 %), while doses of 2.5, 5 and 20 μM were ineffective (65 ± 2 ; 68 ± 2 ; 67 ± 9 % MN preserved, respectively).

ICA-27243 treatment preserves neuromuscular function and slows disease progression in SOD1^{G93A} mice

The neuroprotective effect of ICA-27243 was assessed in female transgenic SOD1^{G93A} mice administered daily at 10 mg/kg/day, compared with SOD1^{G93A} mice receiving vehicle. All the mouse groups maintained a substantial gain in body weight throughout the study. WT and SOD1^{G93A} mice treated with ICA-27243 did not show significant differences in body weight compared to their respective controls, although weight differences are observed between SOD1^{G93A} and WT mice, as expected (Fig. 2A). Additionally, no overt secondary effects were observed in the treated mice as behavior, aggressiveness, fur condition and mobility resembled to untreated littermates.

Electrophysiological tests were conducted to record CMAPs and MEPs of the TA and PL muscles, at the asymptomatic stage (8 weeks), early

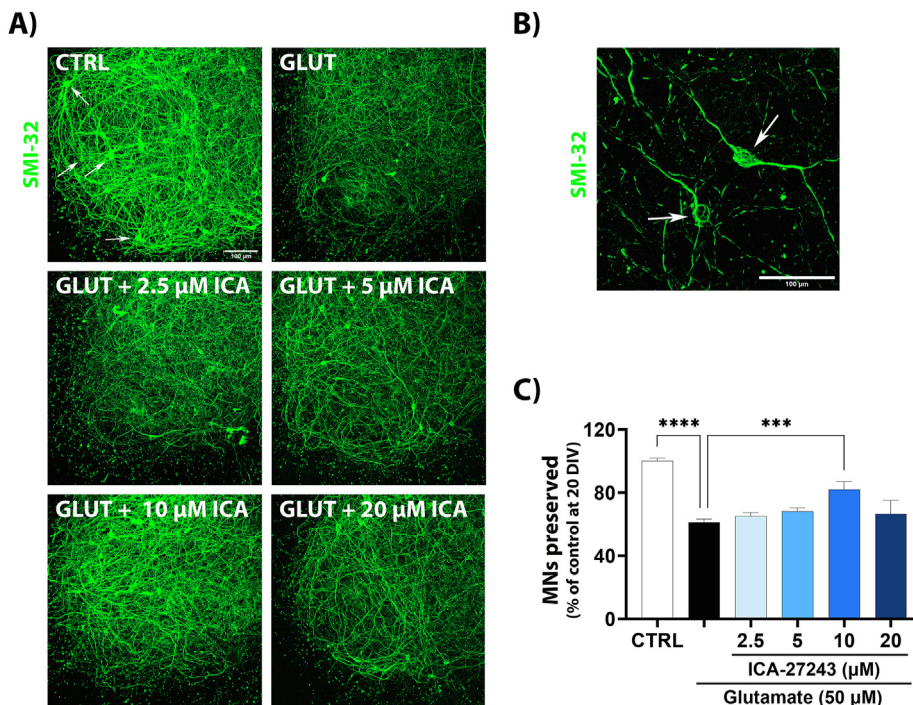


Fig. 1. ICA-27243 prevents MN death in SCOCs exposed to glutamate. (A) Representative confocal images of the ventral horn of spinal cord hemislices labeled for SMI-32, at 20 DIV of tested conditions. White arrows point to MNs soma. Scale bar 100 μm . (B) Representative confocal image magnification of SMI-32⁺ MNs in SCOCs (C) Bar graph showing neuroprotective effect of 10 μM ICA-27243 in SCOCs exposed to glutamate. Data are shown as mean $\pm$ SEM (n = 12–35 hemisections per condition). One-way ANOVA followed by Bonferroni's post hoc test: ****p < 0.0001 and ***p < 0.001 versus Glutamate condition.

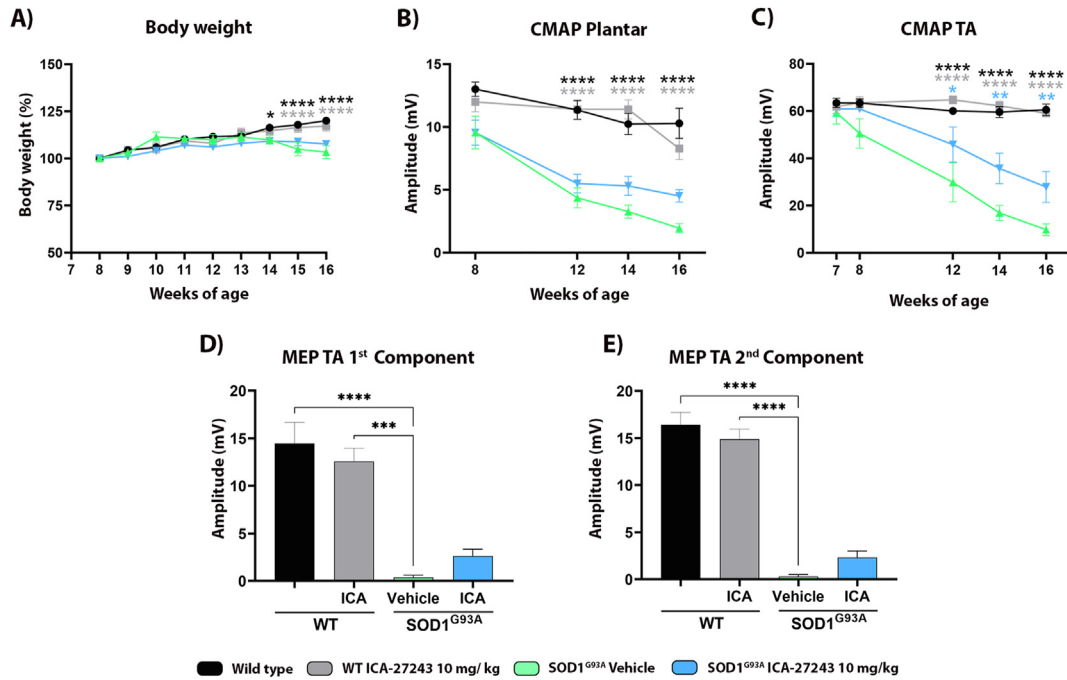


Fig. 2. ICA-27243 treatment enhances neuromuscular function in SOD1^{G93A} mice. (A) Plot showing body weight (expressed as percentage of baseline), from the beginning of the treatment at 8 weeks until 16 weeks of age. (B–C) Plots of the CMAP amplitude of plantar interossei (Plantar) and tibialis anterior (TA) muscles along follow-up. (D) Plots of the amplitude of the first and of the second component of the MEP recorded in the TA muscle. Data are mean $\pm$ SEM, analyzed by One-way (F) and Two-way (A–E) ANOVA followed by Bonferroni's post hoc test. **** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$ vs SOD1^{G93A} vehicle group. Animals per group: WT, $n = 7$; WT + ICA-27243, $n = 6$; SOD1^{G93A} + vehicle, $n = 5$; SOD1^{G93A} + ICA-27243, $n = 6$.

symptomatic stage (12 weeks), mid-symptomatic stage (14 weeks) and at the end of the follow-up (16 weeks). Motor nerve conduction test indicated that ICA-27243 significantly preserved the amplitude of the CMAP of the TA muscle of treated SOD1^{G93A} mice at 12, 14 and 16 weeks of age

(Fig. 2C), whereas it had a low effect preventing the CMAP decline in the PL muscle at late stages of the disease (Fig. 2B), compared to vehicle SOD1^{G93A} mice. Electrophysiological results showed a slower latency to the onset of the M wave of the PL muscle in the SOD1^{G93A} vehicle group

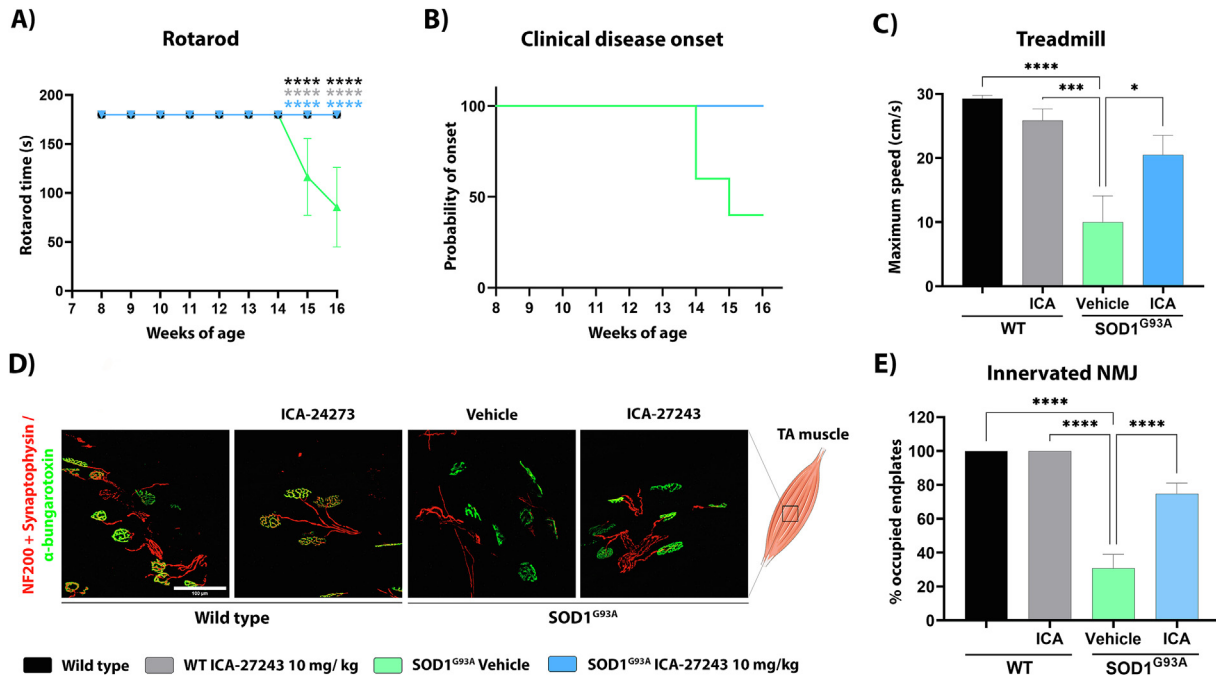


Fig. 3. ICA-27243 administration maintains motor activity in SOD1^{G93A} mice. (A) Graph showing the impact of ICA-27243 treatment on motor function, evaluated by the rotarod test. (B) Clinical disease onset determined as the first day when a decline in performance was observed in the rotarod test. (C) Graph showing the effect of ICA-27243 on locomotion measured as the maximum speed supported on the treadmill test. (D) Representative confocal images of NMJ in the TA muscle of WT and SOD1^{G93A} mice with or without ICA-27243 treatment at 16 weeks of age. Scale bar: 100 μ m. (E) Plot graph showing histological analyses of NMJ of the TA muscle represented as the proportion of occupied endplates at 16 weeks of age. Data are mean $\pm$ SEM. One-way (B,C,E) and Two-way (A) ANOVA followed by Bonferroni's post hoc test: **** $p < 0.0001$; *** $p < 0.001$; * $p < 0.05$ vs SOD1^{G93A} vehicle group.

and SOD1^{G93A} mice treated with ICA-27243, compared to WT groups, as previously demonstrated (Mancuso et al., 2011). However, no differences were found between treated and untreated groups. Similarly, no differences were observed in the M wave duration between all groups. Regarding the MEPs recorded in the TA muscles, SOD1^{G93A} mice treated with ICA-27243 had significantly higher amplitude in the first component and a tendency in the second component compared to vehicle SOD1^{G93A} mice (Fig. 2D–E).

The results of the rotarod test indicated that ICA-27243 treatment significantly improved the functional outcome at both 15 and 16 weeks (Fig. 3A). Thus, the onset of motor impairment in SOD1^{G93A} mice treated with ICA-27243 was delayed by at least two weeks, since vehicle mice exhibited decline in the rotarod from 14 weeks whereas treated mice maintained on the rod for the 180 s until the end of the follow-up (Fig. 3B). In the treadmill test, the performance of SOD1^{G93A} mice was also notably enhanced with administration of ICA-27243 (Fig. 3C). In summary, ICA-27243 treatment showed significant improvement in all tests of motor activity in the SOD1^{G93A} mice.

The assessment of NMJ innervation of the TA muscles at the end of the study showed that ICA-27243 treatment significantly increased the proportion of occupied endplates (75 ± 6 % occupied endplates) compared to untreated SOD1^{G93A} mice (31 ± 8 % occupied endplates). This finding supports the preservation of CMAP amplitudes observed in nerve conduction tests and the improvement in locomotion tests.

ICA-27243 promotes MN survival in the lumbar spinal cord and reduces glial activation in the SOD1^{G93A} mice

The quantification of MNs in the ventral horn of L4–L5 spinal cord slices demonstrated that untreated SOD1^{G93A} mice had only 33 % of surviving MNs (7.4 ± 1 MNs) at 16 weeks of age, compared to untreated WT mice (22.3 ± 0.4 MNs) and WT ICA-27243 treated mice (22.2 ± 0.5 MNs) (Fig. 4A–B). In contrast, SOD1^{G93A} mice treated with ICA-27243 had a significantly higher number of spinal MNs (13.0 ± 0.3 surviving MNs) than untreated SOD1^{G93A} mice, reaching up to 58 % of MN found in WT mice. Both astroglial and microglial reactivity were markedly increased in SOD1^{G93A} mice compared to both groups of WT mice. Treatment with ICA-27243 attenuated glial reactivity, even though it did not reach a significant difference compared to SOD1^{G93A} mice (Fig. 5A–C).

Discussion

The present study demonstrates for the first time that ICA-27243 exhibits neuroprotective effects on spinal cord MNs. *In vitro* ICA-27243 prevented MN loss in SCOCs treated with glutamate to generate excitotoxicity. *In vivo*, ICA-27243 maintained neuromuscular function, enhanced motor activity, reduced the loss of MNs and attenuated glial

activation in the spinal cord of the ALS transgenic SOD1^{G93A} female mice. These findings highlight the interest of compounds modulating neuronal excitability as suitable therapeutic agents.

ICA-27243 was first tested in a SCOC excitotoxic model. The SCOC provides a reliable *in vitro* system for assessing the neuroprotective impact on MNs. It maintains the synaptic connectivity, anatomical organization of the neural circuitry and cellular stoichiometry of both neuronal and non-neuronal elements within the spinal cord slice. This integrated perspective renders SCOC a valuable tool for testing novel drugs for MND therapy before *in vivo* testing [24,25,31]. After the excitotoxic insult, cultures treated with 10 μ M of ICA-27243 presented a higher number of α -MNs, indicating a neuroprotective effect. Since ICA-27243 has no direct action on glutamate receptors, the beneficial results can be interpreted as an enhancement in other protective mechanisms that allow the MNs to respond better to the excitotoxic insult. ICA-27243 is an M-current activator with high selectivity for Kv7.2/7.3, and it can induce a hyperpolarizing shift in the voltage-dependent activation of its target [21]. Furthermore, the addition of KCl, that increases extracellular K⁺, to mouse SCOCs reduces the intrinsic excitability due to the increase of the M-current [32]. Hence, the primary impact of ICA-27243 would be to enhance the M-current, thereby diminishing hyperexcitability and providing an explanation for the neuroprotective effects observed in our model.

To assess the neuroprotective effects in the context of ALS, we tested ICA-27243 in the SOD1^{G93A} transgenic mice, the most widely used ALS preclinical model, since they reproduce most of the clinical and histopathological characteristics of ALS disease (Ripps et al., 1995). We have found that a daily dose of 10 mg/kg of ICA-27243 attenuates the progressive decrease in the CMAP amplitude of hindlimb muscles and, importantly, of the MEPs in the treated compared with the vehicle SOD1^{G93A} mice, indicating effective preservation of the peripheral and the central motor pathways. There were not differences in the latency to the onset and the duration of the M wave, indicating no changes in the conduction velocity of remaining motor fibers [29]. In contrast, treatment of SOD1^{G93A} mice with Riluzole, commonly used in the clinic, was not able to improve the decline of the CMAP amplitude, that was similar to vehicle SOD1^{G93A} mice [30]. Furthermore, our findings revealed that the administration of ICA-27243 resulted in improved outcomes in locomotion tests compared to vehicle SOD1^{G93A} mice. Previous studies have demonstrated the positive effects of ICA-27243 on locomotion in female Sprague-Dawley rats, where a dosage of 10 mg/kg reduced abnormal involuntary movements in L-Dopa-induced dyskinesias [33]. ICA-27243 likely contributes to maintaining the NMJ integrity, probably due to a reduction of the persistent depolarization observed in the motor axon in the SOD1 model [34,35]. This reduction in depolarization is likely achieved through an increase of K⁺ outflow currents. Collectively, ICA-27243 effectively preserves motor function and behavior in the SOD1^{G93A} ALS transgenic mice.

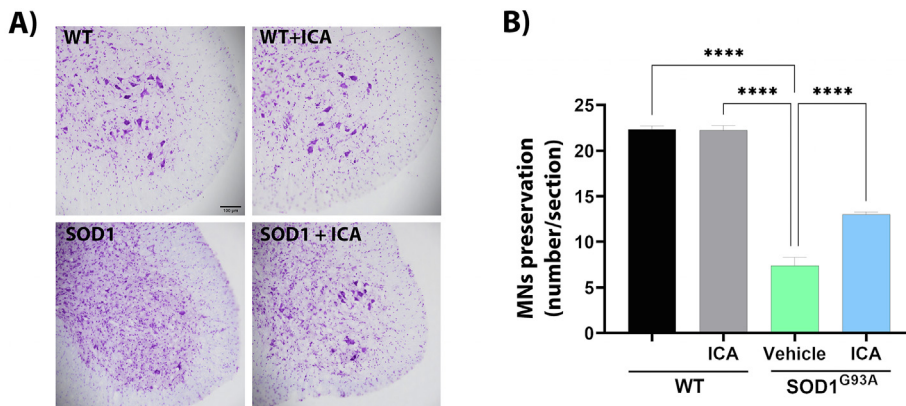


Fig. 4. Neuroprotective effects of ICA-27243 administration on MNs in SOD1^{G93A} mice at 16 weeks. (A) Representative images of the ventral horn of L4–L5 spinal cord stained with Cresyl violet of WT and SOD1^{G93A} mice with or without ICA-27243 treatment at 16 weeks of age. Scale bar 100 μ m. (B) Bar graph showing the neuroprotective effect of 10 mg/kg of ICA-27243, with significantly more MNs per hemisection of the spinal cord than in vehicle SOD1^{G93A} mice. Data are mean $\pm$ SEM. One-way ANOVA followed by Bonferroni's post hoc test: ****p < 0.0001 vs SOD1^{G93A} vehicle group.

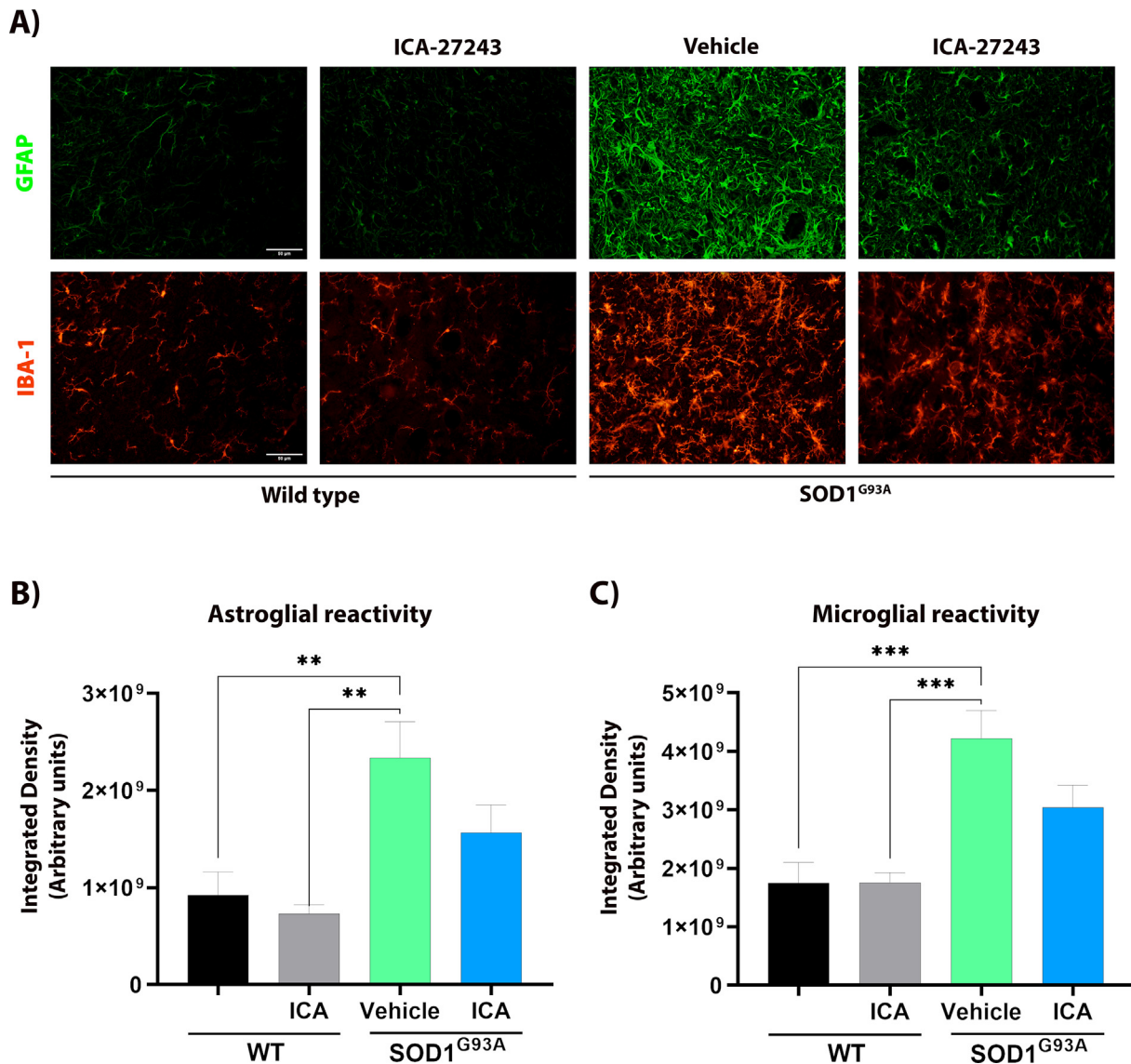


Fig. 5. Administration of ICA-27243 attenuates glial reactivity in SOD1^{G93A} mice at 16 weeks. (A) Representative images of glia immunoreactivity assessed by GFAP and IBA-1 labeling in the ventral horn of L4-L5 spinal cord slices of WT and SOD1^{G93A} mice with or without ICA-27243 treatment at 16 weeks of age. Scale bar: 50 μm. Bar graphs showing astroglial (B) and microglial (C) reactivity represented as the integrated density in the ventral horn of the spinal cord. Data are mean ± SEM. One-way (B–C) ANOVA followed by Bonferroni's post hoc test: ****p* < 0.001; ***p* < 0.01 vs SOD1^{G93A} vehicle group.

Treatment with ICA-27243 significantly prevented MN degeneration in the SOD1^{G93A} mice. As previously discussed, ICA-27243 enhances the M-current reducing excitability. Therefore, we may expect a reduced influx of Ca²⁺ and a downregulation of the Ca²⁺-dependent cascade of cytotoxic events, such as mitochondrial dysfunction or increased ROS levels [36,37]. In fact, a decrease in the production of ROS was observed in cultured hypoglossal MNs treated with Retigabine and DL-threo-β-benzoyloxyaspartate, concomitant with increased MN survival rate, enhancement of the M-current and reduction of hyperexcitability [16]. In a different study, MN survival in ALS-patients iPSC treated with Retigabine was related to ER-stress downregulation [17]. Numerous studies have shown that hyperexcitability, excitotoxicity, oxidative stress and mitochondrial dysfunction are interacting molecular pathways being altered throughout the progression of ALS pathology [2]. In this way, we hypothesize that since Retigabine and ICA-27243 have a common target, the Kv7.2/3 channel, the neuroprotective effect seen in our study with ICA-27243 could be due to modifications in membrane excitability that positively impact on one or more of the molecular pathways mentioned. Although both Retigabine and ICA-27243 increase K⁺ efflux enhancing M-current, their binding sites are different [38], thus, the derived effects

may vary between the two compounds. ICA-27243 specifically enhances the activation Kv7.2/7.3 channels, having little impact on Kv7.5/3 or Kv7.3 channels, whereas Retigabine indiscriminately increases all Kv7 channel currents [38]. Hence, further studies are needed to better understand the neuroprotective properties of ICA-27243 in ALS models.

In addition, we observed that ICA-27243 can attenuate astroglial and microglial reactivity. This action cannot be directly attributable to ICA-27243 effects, since Kv7.2/7.3 channels are predominantly located in the axon initial segment and nodes of Ranvier of neurons [14], and may be secondary to the reduced neuronal death. However, when Retigabine was administered in an experimental autoimmune encephalomyelitis mouse model, the infiltration of IBA-1⁺ cells was decreased [39].

In conclusion, we have demonstrated for the first time that ICA-27243 is an effective treatment that can preserve neuromuscular function, enhance motor activity and prevent MN degeneration in the spinal cord of SOD1^{G93A} mice and in SCOCs. These data highlight that Kv channels, specially Kv7.2/3, represent a potential target for mitigating hyperexcitability in a range of diseases including ALS. The strong evidence here presented is a proof-of-concept for the compound ICA-27243 as a potential therapy for ALS. Therefore, further studies of ICA-27243 on

SOD1^{G93A} male mice and assessment of survival seem guaranteed in order to fully determine the therapeutic potential of this compound.

Author Contribution

Conceptualization: XN, MHG. Execution and analysis: VM. Manuscript preparation: VM, XN, MHG. Review: XN, MHG.

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Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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