Hippocampal synaptic dysfunction in the SOD1<sup>G93A</sup> mouse model of Amyotrophic Lateral Sclerosis: Reversal by adenosine A<sub>2A</sub>R blockade

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HIGHLIGHTS

- Increased synaptic excitability in pre-symptomatic SOD1<sup>G93A</sup> mice hippocampus.
- Impairment of the Long-term potentiation (LTP) and NMDA receptor function in symptomatic SOD1<sup>G93A</sup> mice hippocampus.
- Increased adenosine A<sub>2A</sub> receptor levels at both pre-symptomatic and symptomatic SOD1<sup>G93A</sup> mice hippocampus.
- Prolonged blockade of A<sub>2A</sub> receptors rescues LTP and A<sub>2A</sub>R levels in symptomatic SOD1<sup>G93A</sup> mice.

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) mostly affects motor neurons, but non-motor neural and cognitive alterations have been reported in ALS mouse models and patients. Here, we evaluated if time-dependent biphasic changes in synaptic transmission and plasticity occur in hippocampal synapses of ALS SOD1<sup>G93A</sup> mice. Recordings were performed in hippocampal slices of SOD1<sup>G93A</sup> and age-matched WT mice, in the pre-symptomatic and symptomatic stages. We found an enhancement of pre-synaptic function and increased adenosine A<sub>2A</sub> receptor levels in the hippocampus of pre-symptomatic mice. In contrast, in symptomatic mice, there was an impairment of long-term potentiation (LTP) and a decrease in NMDA receptor-mediated synaptic currents, with A<sub>2A</sub>R levels also being increased. Chronic treatment with the A<sub>2A</sub> receptor antagonist KW-6002, rescued LTP and A<sub>2A</sub>R values. Altogether, these findings suggest an increase in synaptic function during the pre-symptomatic stage, followed by a decrease in synaptic plasticity in the symptomatic stage, which involves over-activation of A<sub>2A</sub>R from early disease stages.

1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is one of the most devastating neurodegenerative disorders and is the most common form of Motor Neuron Disease (MND). In ALS, upper and lower motor neurons degenerate, resulting in progressive loss of neurotransmission and muscle atrophy, ultimately leading to terminal respiratory failure. The widely used ALS animal model, the SOD1<sup>G93A</sup> mouse model, recapitulates most of the human ALS symptomatology, including neuromuscular dysfunction (Naumenko et al., 2011). Pre-symptomatic SOD1<sup>G93A</sup> mice already present signs of neuromuscular transmission impairment (Rocha et al., 2013), highlighting that synaptic dysfunction may start long before the onset of motor symptoms.

Despite being predominantly a motor disease, ALS is now recognized as a multisystem neurodegenerative disease, since non-motor areas of the brain may also undergo degeneration, leading to different degrees of cognitive impairment (Ferrari et al., 2011; Ng et al., 2015; Witgert et al., 2010). Frontotemporal dementia (FTD) is a form of dementia commonly associated with ALS that is frequently, though not necessarily, associated with C9orf72 mutations. How SOD mutations impact non-motor synapses is poorly known. Early changes in synaptic plasticity have been reported to occur in hippocampal slices of pre-symptomatic mice overexpressing the human SOD1<sup>G93A</sup> mutation, namely increased long-term potentiation (LTP) magnitude in Schaffer...
collateral-CA1 synapses, higher hippocampal mRNA expression and protein levels of AMPA subunit GluR1, and improved spatial recognition abilities, altogether suggesting that the excess of glutamate availability, in the pre-symptomatic stage of the disease, may enhance cognitive performance before leading to excitotoxicity (Spalloni et al., 2006). Recently, it was shown that pre-symptomatic SOD1G93A mice present learning and memory impairments accompanied by hippocampal γ-aminobutyric acid (GABA)ergic system dysfunction, further revealing selective deficits antecedent to the onset of motor symptoms (Quarta et al., 2015). However, with the exception of the aforementioned GABAergic dysfunction, comparisons between the degree and type of dysfunction at pre-symptomatic and symptomatic stages were not evaluated in any of these studies.

Adenosine is an endogenous and ubiquitous neuromodulator with a relevant role in pathological and physiological conditions (Sebastião et al., 2018). Through the activation of inhibitory A1 and excitatory A2A receptors (A1R and A2AR, respectively), adenosine regulates synaptic transmission at pre, post- and in-synaptic sites, and modulates the action of other neuromodulators. A2AR activation increases neuronal excitability, leading to excitotoxic and neuroinflammatory effects (Ribeiro et al., 2016), which are hallmarks of ALS. Previous research performed by our lab provided evidence that adenosine receptor modulation is disturbed in ALS. A2AR-mediated excitatory effects on neuromuscular transmission are exacerbated in the pre-symptomatic stage, whereas in the symptomatic stage this excitatory action disappears, suggesting that A2AR function changes over ALS progression (Nascimento et al., 2014). It is known that ALS patients show elevated cerebrospinal fluid adenosine levels (Yoshida et al., 1999), and increased A2AR expression in lymphocytes (Vincenzi et al., 2013) and post mortem spinal cord tissues (Ng et al., 2015). A2AR enhance glutamate release and inhibit glutamate uptake, leading to increased glutamate levels in both the central and peripheral nervous systems, favoring ALS-associated excitotoxicity (Popoli et al., 2003).

Considering previous evidence of early synaptic dysfunction in pre-symptomatic SOD1G93A mice, which are also evident in non-motor areas, namely the hippocampus (Quarta et al., 2015; Spalloni et al., 2006), the first aim of this work was to evaluate time-dependent changes in hippocampal synaptic transmission and synaptic plasticity. We hypothesized that, as observed in motor nerve endings (Nascimento et al., 2015, 2014; Rocha et al., 2013), biphasic alterations could occur in both pre-symptomatic and symptomatic mice. Given the known correlation between hippocampal A2AR upregulation and cognitive and synaptic deficits (Batalha et al., 2013; Cunha, 2016; Kaster et al., 2015; Temido-Ferreira et al., 2018) we then evaluated whether chronic A2AR blockade could prevent synaptic dysfunction.

The data herein reported highlights biphasic synaptic changes in non-motor neurons along ALS progression, with impact in NMDA receptor-mediated current dysfunction and synaptic plasticity impairment, which involve A2AR.

2. Methods

2.1. Ethics statement

All the work reported was performed in accordance with the guidelines of Directive 2010/63/EU, and of the Portuguese Law (DL 113/213). The protocols were approved by the Instituto de Medicina Molecular João Lobo Antunes– iMM’s institutional Animal Welfare Body – ORBEA-iMM and the Portuguese National Authority – DGAV (Direcção Geral de Alimentação e Veterinária).

2.2. Animals

B6SJL-TgN (SOD1-G93A)1Gur/J males expressing the human G93A point mutation (glycine to alanine at residue 93) at SOD1 gene (Cu/Zn superoxide dismutase 1) and wild type (WT) B6SJLF1/J females were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The breeding was performed at iMM rodent facility where a colony was established. SOD1G93A transgenic males were bred with WT females in a rotational scheme. In this study we used SOD1G93A in the pre-symptomatic (4–6 weeks old) and symptomatic stages (16–18 weeks old). Age-matched WT animals were used as controls. Both genders were used. Symptomatic SOD1G93A mice had clear signs of paresis of the hind limbs. Pre-symptomatic mice displayed normal scores in the Rotarod test (Figure Supplementary Fig. 1). Animals were divided into cages (4–5 mice/cage) by gender, and ear tissue was collected to genotype the animals by Polymerase Chain Reaction (PCR) (Rosen et al., 1993). Animals were housed under a 12 h light/12 h dark cycle in a temperature-controlled room (21 ± 1.0 °C) and 55 ± 10% humidity. Animals had ad libitum access to food and water. In each series of data, test and control experiments were performed close in time.

2.3. Field excitatory postsynaptic potentials (fEPSPs) recordings

Acute hippocampal slices were prepared from pre-symptomatic and symptomatic SOD1G93A mice, and from age-matched WT mice, as routinely done in our lab (Jerónimo-Santos et al., 2015). Briefly, after animals were sacrificed by cervical displacement, hippocampi were dissected in ice-cold artificial cerebrospinal fluid (aCSF) solution (composition: 124 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 1 mM MgSO4, 2 mM CaCl2 and 10 mM glucose, pH 7.4) previously gassed with 95% O2 and 5% CO2. Hippocampal slices (400 μm thick), were cut perpendicularly to the long axis of the hippocampus using a McIlwain tissue chopper. Slices were allowed to functionally and energetically recover for at least 1 h in a resting chamber filled with aCSF solution continuously gassed with 95% O2 and 5% CO2. Hippocampal slices were superfused with gassed aCSF solution at a constant flow (3 mL/min) and temperature (32 °C).

Field excitatory postsynaptic potential (fEPSP) recordings were obtained through a microelectrode (4–8 MΩ resistance) filled with aCSF solution, placed in stratum radiatum of CA1 region of the hippocampus. Two pathways of the Schaffer collateral (SC)/commissural fibers were alternately stimulated (rectangular pulses of 0.1 ms duration) every 15 s (for input/output [I/O] curves and paired-pulse facilitation [PPF] recordings) or every 20 s (for LTP recordings), using a bipolar concentric wire electrode. The averages of 6 consecutive responses to the same stimulus intensity delivered to the same pathway were obtained throughout the experiments, and the slope of the initial phase of each averaged fEPSP was quantified. Recordings were obtained with an Axiocamp 2B amplifier (10 Vm output; Axon Instruments, Foster City, CA, USA), which automatically selects 3 dB frequency single pole low-pass filter, digitized (BNC-2110, National Instruments, Austin, TX, USA) at a sampling frequency of 20 KHz (1 per 0.05 ms) and continuously stored on a personal computer with WinLTP software (Anderson and Collingridge, 2001). All the protocols detailed below were started only after a stable baseline of at least 15 min.

Since each slice allows recordings from two independent pathways, the usual protocol was to record first PPF data by stimulation of one pathway, then I/O data by stimulating the second pathway, then post-tetanic potentiation (PTP) and LTP data by again delivering stimulation to the first pathway. While recording fEPSPs for the LTP assays, we kept basal stimulation (one stimulus every 20sec) to the non-theta burst stimulated pathway to allow assessment of the viability of the slice as well as the independence between the two pathways.

2.4. Long term potentiation (LTP) and post-tetanic potentiation (PTP)

0-burst stimulation was used to induce LTP, since this pattern of stimulation is considered closer to what occurs physiologically in the
hippocampus during episodes of learning and memory in living animals (Albens et al., 2007). After obtaining a stable recording of the fEPSP slope, a 0-burst of stimuli, consisting of 1 train of 4 bursts (200 ms interburst interval), each burst being composed by 4 pulses delivered at 100 Hz (1 x (4 x 4)), was applied, and the stimulus paradigm was then resumed to pre-burst conditions until the end of the recording period (60 min after burst stimulation). LTP magnitude was quantified as the % change in the average slope of the fEPSP taken from 50 to 60 min after the induction of LTP as compared with the average slope of the fEPSP measured during the 10 min before LTP induction. PTP was assessed as the average fEPSP slope obtained in the first 4 min after LTP induction (Habets and Borst, 2007).

2.5. Input/output curves

After a stable baseline of at least 15 min, we performed the I/O curves protocol to access synaptic efficiency. We started by decreasing the stimulus delivered to the hippocampal slice until no fEPSP was evoked. Then, we consecutively increased the stimulus from 60 μA to a maximum amplitude of 320 μA, in 20 μA steps, every 4 min. The I/O curves are represented by fEPSP slope against the stimulus intensity, as well as by the fEPSP slope against the amplitude of the pre-synaptic fiber volley.

2.6. Paired-pulse facilitation (PPFs)

To analyse pre-synaptic activity, we performed a protocol consisting in two consecutive stimuli separated by 50 ms, delivered every 15s. The ratio between the slopes of the two consecutive fEPSPs (fEVSSP/I/fEPSP0) was quantified.

2.7. Patch clamp recordings

Experiments were performed in acute transverse hippocampal slices taken from pre-symptomatic and symptomatic SOD1<sup>G93A</sup> mice and age-matched WT mice. Animals were anesthetized with isoflurane before decapitation. Transverse slices (300 μm thick) were obtained with a vibratome (VT1000 S; Leica, Nussloch, Germany) in ice cold dissecting solution containing (in mM) 110 sucrose, 2.5 KCl, 7 MgCl₂, 25 NaHCO₃, 1.25 NaH₂PO₄, and 7.6 glucose, oxygenated with 95% O₂ and 5% CO₂, pH 7.4. Slices were incubated in ACSF solution (composition: 124 mM NaCl, 3 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 1 mM MgSO₄, 2 mM CaCl₂ and 10 mM glucose, pH 7.4) at 35 °C for 30 min and then maintained at room temperature for at least 60 min continuously gassed with 95% O₂ and 5% CO₂. After the recovery period, each slice was transferred to a recording chamber, continuously superfused with ACSF solution at 3 mL/min at room temperature for recording.

Visually guided whole-cell voltage-clamp recordings were performed from CA1 neurons using a Carl Zeiss Axioskop 2FS upright microscope (Jena, Germany) equipped with a differential interference contrast-infrared (DIC-IR) CCD video camera (VX44, Till Photonics, Gräfelfing, Germany) and recorded with an Axopatch 200B (Axon Instruments, Foster City, CA, USA) amplifier. Patch pipettes (4–9 MΩ) were pulled from borosilicate glass capillaries (1.5 mm outer diameter, 0.86 mm inner diameter, Harvard Apparatus, Holliston, MA, USA) with a PC-10 Puller (Narishige Group, London, UK) and filled with an internal solution containing (in mM): 125 CsCl, 8 NaCl, 1 CaCl₂, 10 EGTA, 10 Hepes, 10 glucose, 5 Mg ATP, and 0.4 NaGTP, pH 7.2, adjusted with CaOH₂ (50 wt% in H₂O), 280–290 mOs; for EPSC<sub>AMPA</sub> recordings, 0.1 mM spermine was added.

All recordings were performed in the continuous presence of bicuculline (50 μM) to block GABAergic transmission. Stimuli (0.2 ms rectangular pulses every 15 s) were delivered via monopolar stimulation with a patch-type pipette filled with ACSF and positioned in stratum radiatum. NMDA/AMPA ratios were calculated to check for changes in AMPA or NMDA currents and were obtained from experiments where EPSCs at Vh = −70 mV and Vh = +40 mV were alternately recorded. Peak current amplitude recorded at Vh = −70 mV was taken as an estimate of AMPAR activation. Since currents at Vh = +40 mV are composed of AMPA and NMDA currents, NMDA currents were not measured at peak but at 60 ms after AMPA peak at Vh = −70 mV, where AMPA currents are already negligible.

EPSC<sub>NMDA</sub> and EPSC<sub>AMPA</sub> were recorded in another set of experiments where currents were pharmacologically isolated by adding to the ACSF supplemented with picROTOXIN (50 μM), CNQX (25 μM) or APV (50 μM), respectively. Current/voltage relationships (I/V plots) were obtained from experiments where Vh was alternated from −70 mV to +50 mV (EPSC<sub>AMPA</sub>) or −90 mV to +50 mV (EPSC<sub>NMDA</sub>) with gaps of 10 mV in-between steps. To allow data pooling from different experiments, EPSCs amplitudes were normalized in each experiment by taking as 1 the peak amplitude recorded at −70 mV (EPSC<sub>AMPA</sub>) or +50 mV (EPSC<sub>NMDA</sub>). The rectification index (EPSC(+50/EPSC(-70))mV) for either EPSC<sub>NMDA</sub> or EPSC<sub>AMPA</sub> currents was calculated by the ratio of peak EPSCs amplitudes recorded at a Vh = +50 mV over the peak current amplitude recorded at Vh = −70 mV.

Acquired signals were filtered using an in-built, 2-kHz, 3-pole Bessel filter, and data were digitized at 10 kHz under control of the pCLAMP 11 (Molecular Devices, San José, CA, USA) software program. Offset potentials were nulled before giga-seal formation. Small voltage steps (−5 mV, 50 ms) were used to monitor the access resistance throughout recording and experiments in which this parameter varied by more than 20% were discarded.

2.8. Chronic consumption of KW-6002 (selective A<sub>β</sub>R antagonist)

KW-6002 (istradefylline) was synthesised as previously described (Hockenmeyer et al., 2004) and was diluted in the drinking water as previously in our lab (Mouro et al., 2017). The dose (approx. 3 mg/kg/day, 0.025% methylcellulose) was selected according to previous studies (Yang et al., 2007; Mouro et al., 2017). Water consumption per day per animal was 7–8 mL. The change in body weight throughout the KW-6002 administration period did not exceed 20% (2–4 g from a body weight of about 20 g, Figure Supplementary Fig. 2), for most animals being about 10%. Thus, the concentration of KW-6002 in the drinking water (7.5 μg/mL, corresponding to 19.5 μM) was kept constant throughout the period of drug administration. No marked differences in water consumption were detected while comparing KW-6002 treated mice with control mice.

KW-6002 administration started when animals were 11 weeks old (before overt onset of motor symptoms) and lasted until 16–18 weeks of age, when animals had to be euthanized due to hind limb paralysis. Ex vivo electrophysiological experiments and tissue storage for molecular analysis were performed in the day of euthanasia. The reason for starting treatment when mice were 11 weeks old was to allow treatment onset to be as close as possible to the early symptomatic disease stage, without shortening the treatment too much. At 11 weeks of age SOD1<sup>G93A</sup> mice were not yet clearly symptomatic, regarding hind limb paresis, though some subtle alterations should already be present. In a previous study (Rocha et al., 2013) we detected alterations on the motor profile (assessed by the Rotarod test) of 12–15 weeks old SOD1<sup>G93A</sup> mice. In this paper we decided not to assess motor function of animals that could be close to the symptomatic stage to avoid confounding effects resulting from exercise.

Since KW-6002 is photosensitive, the solution was prepared in a dark chamber and placed into bottles with light protection. The control group received a vehicle solution, containing 0.025% methylcellulose diluted in drinking water. No substantial differences between the two groups (KW-6002 and control) were detected in terms of water intake (approximately 7–8 mL per day per mouse).
2.9. Western blot

Hippocampal samples were homogenized in Ristocetin Induced Platelet Agglutination buffer (RIPA; 50 mM Tris pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 10% glycerol, 1% SDS plus protease inhibitors (Hoffmann LaRochef, Basel, Switzerland)) and then centrifuged. Protein was quantified using a DC™ Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). 50 μL volume of protein extracts for Aβ2R (200 μg of total protein) and 35 μL volume of protein extracts for NR1 NMDAR subunit (70 μg of total protein) from tissue were loaded and separated on 10% SDS-PAGE gels and transferred onto PVDF membranes (Millipore, Burlington, MA, USA). Blots were incubated overnight at 4 °C with primary antibody anti-Aβ2R (1:1500, Cat 05–717, Millipore, Burlington, MA, USA), mouse anti-NMDA receptor subunit 1 (1:500; BD Biosciences, #556308) and anti-GAPDH (1:5000, Ref: AM4300, Invitrogen-Thermo Scientific, Waltham, MA, USA) as a loading control. The membranes were incubated with secondary anti-body anti-mouse, 1:2500 for Aβ2R and 1:10000 for GAPDH (Cat. #172–1101, Bio-Rad Laboratories, Hercules, CA, USA) conjugated with horseradish peroxidase for 1 h at room temperature. For membrane revelation, we used a chemiluminescent detection method with the Amersham-ECL kit (GE Healthcare®, Chicago, IL, USA), and immunoreactivity was visualized using a Chemidoc XRS + system (Bio-Rad Laboratories, Hercules, CA, USA). Western blot densitometry was determined with Image J 1.52a software (National Institutes of Health, Bethesda, MD, USA) and normalized based on band intensity to GAPDH (housekeeping gene).

The Aβ2R antibody was validated against a positive control (WT mouse striatum) and a negative control (Aβ2R Knock out (Aβ2R KO) mouse striatum). The amount of total protein loaded for the both striatum, WT and Aβ2R KO, was 25 μg (Figure Supplementary Fig. 3).

For comparison of pre-symptomatic SOD1G93A and age-matched WT mice, whole hippocampi were used to prepare homogenates. In symptomatic and age-matched WT mice, due to hippocampal slice preparation for fEPSP recordings, we used excess slices for Western Blot.

2.10. Statistical analysis

Data are represented as Mean ± Standard Error of the Mean (SEM) of n independent observations (cells or slices or tissue homogenates from different mice) in each experimental group. One slice per animal was used for each independent protocol of the fEPSP recording experiments, thus n values correspond to number of slices from the same number of mice. For the patch clamp experiments, we recorded from different cells from the same slice, and we used more than one slice of the same mouse; in total of 2–3 different mice of each condition were used (see legend of Fig. 3 for details). For statistical purposes, in the patch clamp experiments, n values correspond to the number of cells since variability between different cells from the same slice is not appreciably different from that between slices from the same animal or between animals of the same group. In the Western Blot experiments, n values correspond to the number of samples from different mice (n = 5), which is equal to the number of blots analyzed per condition, since each well of the Western Blot gel was loaded with a sample from a single mouse. Normality was assessed through the Shapiro-Wilks test, as well as by visual inspection of quantile-quantile plots. Unpaired two-tailed Student’s t-tests for independent samples were used to performed two-sample comparisons; two-way ANOVA followed by Holm-Sidak correction for multiple comparisons were used for the comparison between more than two groups. Values of p < 0.05 were considered statistically significant. Statistical analysis was performed with the GraphPad Prism 8 software (San Diego, CA, USA).

3. Results

3.1. Impaired long-term potentiation (LTP) in symptomatic, but not in pre-symptomatic SOD1G93A mice

To address synaptic plasticity alterations in the hippocampus of SOD1G93A mice at different disease stages, LTP was assessed in the CA1 area of hippocampal slices taken from pre-symptomatic and symptomatic SOD1G93A mice, as well as aged-match WT mice. As expected, 0-burst stimulation of the Schaffer-collateral pathway led to an initial enhancement of the fEPSP slope, followed by a decrease of the slope towards a stabilization above the values recorded before 0-burst stimulation (baseline). LTP magnitude, quantified as the % change in the average slope of the fEPSP before and 50–60 min after 0-burst stimulation (see Methods) was 41.5 ± 9.41% in pre-symptomatic SOD1G93A mouse (n = 6 slices from 6 different mice) and 44.2 ± 9.95% in the age-matched WT mouse (n = 6 slices from 6 different mice). In both cases, the enhancement of fEPSP slope was significantly different from zero (WT = t(5) = 4.44, p = 0.007, n = 6 slices from 6 different mice; pre-symptomatic SOD1G93A = t(5) = 4.41, p = 0.007, n = 6 slices from 6 different mice) indicating that LTP was effectively induced. Comparison of LTP magnitude in both genotypes revealed no statistically significant differences (t(10) = 0.2, p = 0.85, unpaired Student’s t-test; Fig. 1A and B) indicating that pre-symptomatic SOD1G93A mice have no alterations in synaptic plasticity parameter.

Hippocampal slices from symptomatic SOD1G93A mice also displayed LTP (Fig. 2A). However, in contrast with what was observed at the pre-symptomatic stage, LTP magnitude in symptomatic SOD1G93A mice (18.8 ± 4.32%, n = 9 slices from 9 different mice) was significantly lower (t(13) = 3.07, p = 0.009, unpaired Student’s t-test; Fig. 2A and B; n = 9 slices from 9 different mice) than that obtained in slices from age-matched WT mice (44.8 ± 8.20%, n = 6 slices from 6 different mice), highlighting an impairment of hippocampal synaptic plasticity during the symptomatic stages of ALS.

Neither pre-symptomatic (WT = 107 ± 30.0%, n = 6 slices from 6 different mice, pre-symptomatic SOD1G93A = 91.0 ± 15.4%, n = 6 slices from 6 different mice; t(10) = 0.489, p = 0.635, unpaired Student’s t-test; Fig. 1C) nor symptomatic SOD1G93A mice (WT = 117 ± 25.1%, n = 6 slices from 6 different mice; symptomatic SOD1G93A = 70.4 ± 12.1%, n = 9 slices from 9 different mice; t(13) = 1.87, p = 0.084, unpaired Student’s t-test; Fig. 2C) showed statistically significant differences in PTP magnitude.

3.2. Synaptic responses to strong stimulation are increased in pre-symptomatic, but not in symptomatic SOD1G93A mice

To assess alterations in basal synaptic efficiency, I/O curves were recorded from the Schaffer collateral-commissural fibers of the hippocampus (see methods). We observed that the synaptic responses to different stimulation were increased in pre-symptomatic but not symptomatic mice. Indeed, the maximum fEPSP slope (fEPSP top parameter), obtained by non-linear fitting of the data (not by direct measurement of the highest value), was higher in hippocampal slices from pre-symptomatic SOD1G93A mice than from age-matched WT mice (Fig. 1D), suggesting an increase in synaptic responses to strong stimulation (WT = 1.45 ± 0.104 mV/ms, n = 8 slices from 8 different mice; pre-symptomatic SOD1G93A = 2.30 ± 0.176 mV/ms, n = 5 slices from 5 different mice; t(11) = 4.49, p = 0.0009, unpaired Student’s t-test; Fig. 1D, inset). Presynaptic fiber volley (PSFV) amplitude was not appreciably different between WT and pre-symptomatic mice, as demonstrated by the PSFV amplitude elicited with the strongest stimulus (PSFV top parameter; WT = 1.21 ± 0.218 mV, n = 8 slices from 8 different mice; pre-symptomatic SOD1G93A = 1.3 ± 0.59 mV, n = 5 slices from 5 different mice; t(11) = 0.18, p = 0.861, unpaired Student’s t-test; Fig. 1E, inset), suggesting an increase in synaptic efficiency (Fig. 1F) in the absence of major changes in the recruitment of
presynaptic neurons.

I/O curves obtained from experiments using symptomatic SOD1<sub>G93A</sub> mice and age-matched WT mice revealed no differences either in the fEPSP top parameter (WT = 2.19 ± 0.379 mV/ms, n = 7 slices from 7 different mice; symptomatic SOD1<sub>G93A</sub> = 2.42 ± 0.379 mV/ms, n = 6 slices from 6 different mice; t(11) = 0.415, p = 0.681, unpaired Student’s t-test; Fig. 2D, inset) or in PSFV top parameter.

Fig. 1. Pre-synaptic activity, but not LTP, is altered in the hippocampus of pre-symptomatic SOD1<sub>G93A</sub> mice. (A) top panel: time course change of fEPSP slope change (%) caused by θ-burst stimulation (arrow) used to induce long-term potentiation (LTP) in hippocampal slices taken from pre-symptomatic (4–6 weeks old) SOD1<sub>G93A</sub> mice and age-matched wild type (WT) mice. LTP was induced in one pathway (PLTP) while responses to standard stimulation in a second pathway (P<sub>0</sub>) were recorded to assess slice viability and recording stability. fEPSP slope values are normalized to the average fEPSP slope recorded for 10 min before inducing LTP; below the time course panel are shown representative traces of fEPSPs (preceded by the stimulus artefact and the pre-synaptic volley) recorded before (1 and 3) and after (2 and 4) inducing LTP in slices from WT (1 and 2) or SOD1<sub>G93A</sub> (3 and 4) mice; tracings recorded from the same slice are superimposed; (B) Comparison of LTP magnitudes obtained in the experiments illustrated in (A) (see methods for further details). (C) Comparison of the magnitude of post-tetanic potentiation (PTP) observed in the experiments illustrated in (A). (D) input/output (I/O) curves where the fEPSP slope values (ordinates) recorded from SOD1<sub>G93A</sub> mice and age-matched WT mice were plotted against the intensity of stimulation (abscissae); inset: top parameter of the fitted curves was obtained by best fit (GraphPad program) of the I/O curves; note the significant increase in the top parameter value in slices from SOD1<sub>G93A</sub> mice as compared with WT mice; (E) presynaptic fiber volley (PSFV) amplitude from SOD1<sub>G93A</sub> mice and age-matched WT mice plotted as a function of stimulus intensity; inset: top parameter of the fitted curves, which was not significantly different between genotypes. (F) fEPSP slope from SOD1<sub>G93A</sub> mice and age-matched WT mice is plotted as a function of a pre-synaptic fiber volley (PSFV) amplitude. (G) data from paired-pulse facilitation (PPF) experiments; in the left panel the ordinates represent the ratio of fEPSP slopes induced by the 2nd (S1) over the first (S0) stimulation (see methods for further details); representative tracings of paired fEPSPs recorded from hippocampal slices of WT (upper) or SOD1<sub>G93A</sub> mice (lower) are shown in the right panel; note the decrease in PPF in slices from SOD1<sub>G93A</sub> mice, which suggests a pre-synaptic facilitation of transmission. In A-G, data are expressed as mean ± SEM; n = 5–9 slices from different animals; *p < 0.05, **p ≤ 0.001; unpaired Student’s t-test.
3.3. Pre-synaptic function is increased in pre-symptomatic, but not in symptomatic SOD1<sup>G93A</sup> mice

Paired-pulse facilitation (PPF) can be used as a way to assess pre-synaptic modifications at the Schaffer collateral-CA1 synapses, such that decreases in PPF are associated with increases in the probability of glutamate release and in the quantal content of the EPSPs (McNaughton, 1982). As such, we used a PPF protocol with 50 ms interval (Foster and McNaughton, 1991), and quantified the averaged ratio between the slope of two consecutive fEPSPs (see methods) in hippocampal slices of both pre-symptomatic and symptomatic SOD1<sup>G93A</sup> mice and age-matched WT mice. There was a significant decrease of PPF (fEPSP<sub>1</sub>/fEPSP<sub>0</sub>) in hippocampal slices of pre-symptomatic mice when compared with age-matched WT mice (WT = 1.66 ± 0.069, n = 9 slices from 9 different mice; SOD1<sup>G93A</sup> = 1.32 ± 0.160, n = 6 slices from 6 different mice; t(13) = 2.16, p = 0.0497, unpaired Student’s t-test; Fig. 1G) suggesting a presynaptic increase in glutamate release, previous to the appearance of symptoms.
of the motor symptoms of ALS. These changes were no longer observed in symptomatic mice when compared to age-matched WT mice (WT = 1.64 ± 0.128, n = 7 slices from 7 different mice; symptomatic SOD1G93A = 1.58 ± 0.060, n = 8 slices from 8 different mice; t (13) = 0.468, p = 0.648, unpaired Student’s t-test; Fig. 2G).

3.4. Increased AMPA-NMDA ratio in symptomatic SOD1G93A mice

To further dissect the synaptic changes that may account for the LTP alterations observed in symptomatic SOD1G93A mice, AMPA and NMDA receptor function was evaluated through patch-clamp recordings. We
first evaluated alterations in AMPA/NMDA receptor ratio and observed that the ratio was significantly increased in SOD1G93A mice when compared with age-matched WT mice (WT = 1.67 ± 0.207, n = 11 slices from 3 different mice; symptomatic SOD1G93A mice: 3.82 ± 0.538, n = 12 slices from 2 different mice; t(21) = 3.6, p = 0.002, unpaired Student’s t-test; Fig. 3A). The increase in the relative ratio between AMPA and NMDA receptor-mediated currents may be attributable to various factors: an increase in AMPA receptor number/function, a decrease in NMDA receptor number/function or a change in both. Therefore, we performed current–voltage (I–V) relationships in pharmacologically isolated AMPA receptor and NMDA receptor responses.

The AMPA receptor I/V relationship was performed in the presence of APV (50 μM) to isolate AMPA component of the EPSCs. The voltage-dependency of AMPA receptor activation (normalized in each cell by the amplitude at −70 mV to allow comparison between different cells, see Methods) was similar in symptomatic and age-matched WT mice. Current rectification at positive potentials (EPSC +50 mV/EPSC −70mV) was also not changed (WT rectification index: 0.605 ± 0.142, n = 4 slices from 2 different mice; symptomatic SOD1G93A rectification index: 0.489 ± 0.105; t(9) = 0.605, n = 7 slices from 2 different mice, p = 0.559, unpaired Student’s t-test; Fig. 3B and C).

On the other hand, the voltage-dependency of NMDA receptor activation, recorded in the presence of CNQX (25 μM) to isolate the NMDA component of the EPSC, was altered when comparing data from symptomatic SOD1G93A animals and age-matched WT mice. The current magnitude recorded for each voltage value (normalized in each cell for the amplitude at +50 mV, to allow comparison between different cells, see Methods) was lower in pyramidal neurons in slices of symptomatic SOD1G93A mice than in age-matched WT mice, a difference that was maximal when NMDA currents where maximal, i.e. when Vh was set at −30mV (Fig. 3D). Also, current rectification at negative holding potentials calculated by rectification index between EPSC +50 mV and EPSC −70mV, was significantly increased (WT rectification index: 1.42 ± 0.137, n = 12 slices from 3 different mice; symptomatic SOD1G93A rectification index: 2.35 ± 0.319, n = 10 slices from 3 different mice; t(23) = 2.84, p = 0.010, unpaired Student’s t-test, Fig. 3E), indicating decreased NMDA receptor responses in neurons recorded from symptomatic SOD1G93A animals.

When recording I/V relationship of NMDA receptor-activated currents from hippocampal slices of pre-symptomatic SOD1G93A mice, no changes in current rectification curves were observed between WT and SOD1G93A animals (Fig. 3F). The rectification index between EPSC +50 mV and EPSC −70mV was also not statistically different (WT rectification index: 2.31 ± 0.250, n = 11 slices from 3 different mice; pre-symptomatic SOD1G93A rectification index: 2.25 ± 0.313, n = 14 slices from 3 different mice; symptomatic SOD1G93A rectification index: 2.35 ± 0.319, n = 10 slices from 3 different mice; t(23) = 0.15, p = 0.882, unpaired Student’s t-test, Fig. 3G), indicating similar NMDA receptor response in neurons recorded from control and pre-symptomatic SOD1G93A animals.

Taken together, these results reveal an increased AMPA/NMDA ratio in hippocampal pyramids from symptomatic SOD1G93A mice, which may explain the changes observed in LTP in these animals, which might be primarily attributable to decreased NMDA receptor function and/or number rather than to AMPA receptor dysfunction. These alterations could not be detected in pre-symptomatic mice, suggesting that postsynaptic modifications in glutamatergic receptor function do not occur in the very first steps of synaptic dysfunction.

3.5. Chronic blockade of adenosine A2A receptors prevents LTP dysfunction in symptomatic SOD1G93A mice

The above data point towards an early enhancement of glutamate release in SOD1G93A mice, followed by a later postsynaptic dysfunction of NMDA receptors accompanied by synaptic plasticity impairments. Adenosine A2A receptor blockade reverses the deleterious effects upon LTP observed in symptomatic SOD1G93A mice, without affecting short term forms of plasticity, as PPF or PPF, which transmission, an action that gains particular relevance under pathological conditions (Cunha, 2016; Ribeiro et al., 2016). Early enhancement of pre-synaptic activity followed by late synaptic dysfunction was also detected at the neuromuscular junction of SOD1G93A mice (Rocha et al., 2013), which correlates with an early gain of function of excitatory adenosine A2A receptor blockers (Nascimento et al., 2014). Furthermore, a relationship between hippocampal A2A receptor upregulation and synaptic dysfunction has been consistently reported (Batalha et al., 2013; Cunha, 2016; Temido-Ferreira et al., 2018). To address the hypothesis that enhanced A2A receptor activity could account for the synaptic dysfunctions detected in the symptomatic SOD1G93A mice, we treated mice with an orally active A2A receptor antagonist, KW-6002, and later assessed hippocampal synaptic plasticity dysfunctions. The dose of KW-6002 (3 mg/kg/day) was selected according to previously tested selectivity and efficacy of this drug after oral administration (Yim et al., 2008). We used four different groups of animals, which were treated from the age of 11 weeks onwards: WT mice treated with vehicle (WT-VEH; 0.025% methylcellulose), WT mice treated with KW-6002 (WT-KW; 3 mg/kg/day), SOD1G93A mice treated with vehicle (SOD-VEH), and SOD1G93A mice treated with KW-6002 (SOD-KW). Treatment was maintained for 5–7 weeks until LTP assessments in hippocampal slices taken from mice with 16–18 weeks of age. The animals from the four different groups were weighed every two days during KW-6002 treatment and until sacrifice (Figure Supplementary Fig. 2) and no differences in weight gain were observed between the four groups. Two-way ANOVA of LTP magnitude found significant main effects of both genotype (F(1,37) = 4.6; p = 0.039) and treatment (F(1,37) = 8.41, p = 0.006), as well as a significant genotype × treatment interaction (F(1,37) = 5.59, p = 0.023). Post-hoc comparisons confirmed that LTP magnitude was decreased in vehicle-treated SOD1G93A mice in comparison to vehicle-treated WT mice (WT-VEH = 39.3 ± 5.75%, n = 14 slices from 14 different mice; SOD-VEH = 7.38 ± 5.58%, n = 15 slices from 15 different mice; p = 0.001). Remarkably, KW-6002 treatment in SOD1G93A effectively recovered LTP magnitude to vehicle-treated WT levels (SOD-KW = 44.6 ± 7.11%, n = 6 slices from 6 different mice; p = 0.935; Fig. 4A and B). Furthermore, while in SOD1G93A a significant difference was observed between vehicle- and KW-6002-treated animals (p = 0.003; n = 15 slices from 15 different mice for SOD-VEH condition and n = 6 slices from 6 different mice for SOD-KW condition), no such effects were observed for KW-6002-treated WT mice when compared to vehicle-treated WT mice (WT-KW = 43.0 ± 7.30%, n = 6 slices from 6 different mice; WT-VEH = 39.3 ± 5.75%, n = 14 slices from 14 different mice, p = 0.935).

Regarding PPF values, no significant main effects of either genotype (F(1,37) = 2.81, p = 0.102) or treatment (F(1,37) = 0.196, p = 0.660) were found. Likewise, no significant interaction between genotype and treatment (F(1,37) = 0.482, p = 0.827) was found. Post-hoc tests confirmed these results, such that no differences were found in any comparison (WT-VEH = 121 ± 12.6%, n = 14 slices from 14 different mice; WT-KW = 125 ± 23.6%, n = 6 slices from 6 different mice; SOD-VEH = 85.0 ± 16.1%, n = 15 slices from 15 different mice; SOD-KW = 97.5 ± 20.7%, n = 6 slices from 6 different mice; p ≥ 0.425 for every comparison; Fig. 4C).

Finally, concerning PPF, analysis revealed no significant main effects of either genotype (F(1,28) = 0.07905 p = 0.7807) or treatment (F(1,28) = 1.10, p = 0.304), nor a significant interaction between the two (F(1,28) = 1.02, p = 0.321). Congruently, no differences were found in any of the post-hoc comparisons performed (WT-VEH = 1.64 ± 0.161, n = 8 slices from 8 different mice; WT-KW = 1.40 ± 0.092, n = 10 slices from 10 different mice; SOD-VEH = 1.49 ± 0.130, n = 6 slices, from 6 different mice; SOD-KW = 1.49 ± 0.074, n = 8 slices from 8 different mice; p > 0.5 for all comparisons; Fig. 4D).

Altogether, these data indicate that KW-6002 treatment reverses the deleterious effects upon LTP observed in symptomatic SOD1G93A mice, without affecting short term forms of plasticity, as PPF or PPF, which
SOD1G93A mice and age-matched wild type (WT) mice, treated or not with KW-6002, we tested whether the LTP impairment observed in symptomatic SOD1G93A mice, we found no significant differences in immunoreactivity between any of the four groups (p = 0.999 for all comparisons; two-way ANOVA with Holm-Sidak correction for multiple comparisons, n = 5 samples from 5 different mice in each group; Figure Supplementary Fig. 4). As such, these results suggest that, while LTP impairment observed in symptomatic SOD1G93A mice was associated with a decrease in NMDAR-mediated synaptic currents, neither these changes – nor the observed rescuing effect of A2AR blockade over impaired LTP – are related to changes in NMDAR protein levels.

3.6. A2AR levels are increased in hippocampus of both pre-symptomatic and symptomatic SOD1G93A mice

Western blot assays in hippocampal tissue obtained from pre-symptomatic SOD1G93A mice and age-matched WT mice, revealed a significant increase in A2AR protein levels in the former group (WT = 1.00 ± 0.093, n = 5 samples from 5 different mice; pre-symptomatic SOD1G93A = 1.46 ± 0.033, n = 5 samples from 5 different mice; (8) = 4.7; p = 0.002; Fig. 5A).

To compare hippocampal A2AR protein levels in symptomatic SOD1G93A mice, and the putative effect of KW-6002-treatment on these levels (Fig. 5B), a two-way ANOVA was performed. While the analysis revealed no significant main effect of genotype (F(1,16) = 1.54, p = 0.233), a significant main effect was found for treatment (F(1,16) = 7.83, p = 0.013). Moreover, a significant genotype by treatment interaction was also observed (F(1,16) = 9.89, p = 0.006). Post-hoc comparisons revealed an increase of A2AR protein levels in the hippocampus SOD1G93A mice when compared to age-matched WT mice (WT-VEH = 1.00 ± 0.072, n = 5; SOD-VEH = 1.55 ± 0.158, n = 5 samples from 5 different mice; p = 0.045). Notably, KW-6002 treatment restored A2AR protein levels to control levels in SOD1G93A mice (SOD-KW = 0.739 ± 0.158, n = 5 samples from 5 different mice; p = 0.481), while having no effect in WT mice (WT-KW = 0.952 ± 0.093, n = 5 samples from 5 different mice; p = 0.809). Congruently, comparison of VEH- and KW-6002-treated SOD1G93A mice, showed that KW-6002 treatment led to a significant reduction in hippocampal A2AR levels (p = 0.004). Furthermore, no difference was found, with regards to A2AR protein levels, between KW-6002-treated WT- and SOD1G93A mice (p = 0.491, n = 5 samples from 5 different mice in all tested groups).

4. Discussion

The main finding of this study is that in an animal model of ALS, the SOD1G93A mouse, non-motor synapses undergo biphasic changes in synaptic transmission and plasticity, which can be mitigated by oral administration of a drug that targets excitotoxicity. We found early alterations in PPF, compatible with an early enhancement of pre-synaptic function in the pre-symptomatic stage of the disease, followed by an impairment of LTP in symptomatic animals, accompanied by decreased NMDA receptor function. Importantly, the levels of a neuromodulatory receptor, the excitatory A2AR, were found to be already increased in the pre-symptomatic stage. In vivo KW-6002 administration, starting at post-natal week 11 – that is, at an age where synaptic alterations but not motor symptoms already exist – reverted the synaptic plasticity impairments in the symptomatic stage of the disease. KW-6002 is orally active and has already been approved for use in humans for other neurodegenerative diseases. Cortical hyperexcitability preceding the onset of clinical motor symptoms has been reported in familial and sporadic cases of ALS (Vucic et al., 2008; Vucic and Rutkove, 2018), and has been pointed out as having diagnostic utility (Vucic and Rutkove, 2018). Therefore, our data, showing that a drug administered orally at an early disease stage can prevent further synaptic dysfunctions in later disease stages, opens novel possibilities...
for refraining synaptic impairment in ALS.

Our findings of impaired LTP in SOD1<sup>G93A</sup> mice are in line with studies showing that cognitive impairment is a component of the pathological spectrum of ALS, being detected not just in patients with mutations associated with FTJD, but also in non-carrier patients (Poerster et al., 2012; Strong and Yang, 2011; Wilson et al., 2001; Witgert et al., 2010). The recently developed animal model of FTJD, the C9orf72 BAC mouse model, which combines motor deficits and neurodegenerative features of ALS/FTJD, including cortical and hippocampus degeneration and TDP-43 inclusions (Liu et al., 2016), was considered inappropriate for the present study because not all C9orf72 BAC mice develop the disease. This fact complicates the assessment of changes in pre-symptomatic disease stages, as well as the development of drugs to prevent these symptomatic alterations in the prodromal stage of the disease.

The presently reported non-motor synaptic dysfunctions in SOD1<sup>G93A</sup> mice, an ALS model usually not considered to have other than motor symptoms, are in line with previous reports of molecular and synaptic changes at the hippocampal synapses of SOD1<sup>G93A</sup> mice (Quarta et al., 2015; Spalloni et al., 2006). It has also been shown that endogenous glutamate levels are increased in the cerebral cortex of SOD1<sup>G93A</sup> mice (Alexander et al., 2000; Andreasen et al., 2001). Moreover, Spalloni and colleagues (Spalloni et al., 2006) demonstrated that GluR1 protein levels were increased in the hippocampus of pre-symptomatic SOD1<sup>G93A</sup> mice, in association with increased LTP at Schaffer collateral-CA1 fibers of the hippocampus, and spatial learning improvements. We have not observed significant differences in LTP magnitude between pre-symptomatic and age-matched control animals. These differences may be related with the age of the animals, since we tested the animals at a very early stage of disease progression (4–6 weeks of age) while Spalloni and colleagues used older mice, with 90–100 days of age, corresponding to the period just before the onset of clinical symptoms. Notwithstanding the absence of LTP alterations at this early stage (4–6 weeks of age), alterations in synaptic transmission were already evident. Indeed, synaptic transmission in response to strong stimulation was significantly enhanced and PPF was decreased in pre-symptomatic mice, when compared with age-matched controls indicating that, well before the onset of symptoms, an enhancement of excitatory transmission and glutamate release already exists.

The abnormally enhanced glutamatergic function in pre-symptomatic disease stages (present work; Spalloni et al., 2006), associated with a decrease in GABAergic transmission (Nieto-Gonzalez et al., 2011; Quarta et al., 2015) may represent a compensatory function of healthy neurons/synapses to offset a few degenerating ones. This may, however, exacerbate excitotoxicity, leading to later synaptic dysfunctions, including LTP dysfunction herein detected in the symptomatic stage of the disease. In some models of neurodegenerative diseases, like Parkinson’s Disease, or even in early aging conditions, hyperexcitability associated with early enhancement of NMDA receptor function has been proposed as a trigger for later synaptic dysfunction, including dysfunctions in synaptic plasticity (Diojenes et al., 2012; Diojenes et al., 2011; Ferreira et al., 2017; Tanqueiro et al., 2018). However, NMDA receptor function was not altered in the pre-symptomatic SOD1<sup>G93A</sup> mice, rendering it unlikely that early NMDA receptor overactivation is causally related to the LTP impairment detected in these animals.

NMDA receptors are under the control of adenosine A<sub>2</sub>A<sub>R</sub>, which regulate their gating (Tebano et al., 2005; Temido-Ferreira et al., 2018), synaptic activity (Mouro et al., 2018), and desensitization (Sarantis et al., 2015). The results here described suggest that the enhanced A<sub>2</sub>A<sub>R</sub> levels and signaling, detected in SOD1<sup>G93A</sup> mice (present results (Ng et al., 2015; Potenza et al., 2013); and ALS patients (Vincenzi et al., 2013; Yoshida et al., 1999), together with excessive glutamate (Rothstein et al., 1990; Shaw et al., 1995), may play a crucial role in synaptic dysfunctions in ALS, exacerbating excitotoxicity, consequently leading to NMDA receptor dysfunctions and LTP impairments in later disease stages. However, this functional impairment of the NMDAR in the symptomatic SOD1<sup>G93A</sup> mice, was not associated with a decrease of NRI NMDAR protein levels on the hippocampus.

Glutamatergic transmission in the hippocampus is under control of adenosine A<sub>2</sub>A<sub>R</sub>, which is released by the pre- and post-synaptic components, as well as from glial cells, having a neuroprotective and homeostatic role in neurons and synapses in physiological conditions (Dias et al., 2013). By acting pre-synaptically and post-synaptically, A<sub>2</sub>A<sub>R</sub> at the forebrain: 1) enhance glutamate release (Popoli et al., 2003); 2) facilitate AMPA (Dias et al., 2012) and synaptic NMDA receptor mediated responses (Mouro et al., 2018); 3) inhibit glutamate uptake (Matos et al., 2012); 4) dis inhibit glutamatergic transmission, by inhibiting inhibitory inputs to PV-containing neurons (Rombo et al., 2015); 5) facilitate NMDA receptor gating (Tebano et al., 2005; Temido-Ferreira et al., 2018) and 6) modulate NMDA receptor desensitization (Sarantis et al., 2015). Despite the fact that, at non-pathological conditions, the
facilitatory actions of A2AR favor LTP (Dias et al., 2012; Rebola et al., 2008), in pathological conditions A2AR overactivation may lead to synaptic plasticity dysfunctions (Costenla et al., 2011; Diçgenes et al., 2011; Temido-Ferreira et al., 2018). Several studies have shown that adenosine levels are elevated in cerebrospinal fluid of ALS patients (Yoshida et al., 1999). Moreover, A2AR levels are highly increased in SOD1G93A spinal motor neurons and in post-mortem human ALS patient samples (Ng et al., 2015), as well as in the hippocampus of SOD1G93A mice even at early pre-symptomatic stages (present work). The increase of A2AR receptor levels in pre-symptomatic mice probably occurs at the presynaptic side, since no alteration of LTP were detected, while an increase of maximal values of I/O curves and decrease of PPF values occurred. However, since A2AR increase glutamate release (Canas et al., 2018; Lopes et al., 2002) and decrease glutamate transport (Matos et al., 2013), the increase in A2AR protein levels likely creates conditions for increased excitotoxicity, which may attain particular relevance ALS. A2AR blockade prevented this increase in A2AR levels, which was somehow unexpected since antagonists, if anything, are expected to cause an upregulation of receptors. One may speculate that enhanced excitability during the pre-symptomatic stage leads to enhanced release of endogenous substances that lead to further overexpression of A2AR that lead to further excitability, thus creating a positive feedback loop towards excitotoxicity. It is likely that A2AR antagonists by cutting that loop mitigate further impairments of synaptic signalling. By showing that prolonged treatment with the A2AR antagonist, KW-6002, prevented the LTP impairment observed in symptomatic SOD1G93A mice, our data is in full agreement with this interpretation. It is worthwhile to note that, in spite of KW-6002 being administered at a stage where synaptic overactivity was already evident, and A2AR protein levels were noted, that, in spite of KW-6002 being administered at a stage where synaptic overactivity was already evident, and A2AR protein levels were already increased, it not only prevented LTP impairment but also reversed the increases in A2AR protein levels in the symptomatic mice. Indeed, while KW-6002-treated WT mice had similar A2AR levels to those of non-treated WT mice, indicating absence of effect of the A2AR antagonist upon A2AR expression in non-diseased animals, the KW-6002-treated symptomatic SOD1G93A mice had A2AR levels identical to WT mice and much lower than those detected in vehicle-treated symptomatic SOD1G93A mice. If anything, a receptor antagonist would be expected to cause up- and not downregulation of receptor expression. Altogether, our results strongly suggest that the increased activity of excitatory synapses leads to enhanced A2AR expression, further leading to increases in excitability and A2AR expression, towards excitotoxicity. KW-6002, by preventing the A2AR-mediated enhancement of excitability, stops this vicious cycle, restoring A2AR levels, thus preventing excitotoxicity and synaptic plasticity dysfunction, in the symptomatic disease stages. Previous attempts using caffeine, a non-selective A1R and A2AR antagonist, resulted in either disappointing toxicity – exacerbating disease consequences (Potenza et al., 2013) – or absence of risk or benefit in ALS (Fondell et al., 2015; Petimar, 2019). Weather these findings result from the simultaneous blockade of neuroprotective A1R and excitotox A2AR is unknown. However, blockade of A1R alone, starting at the pre-symptomatic phase (70 days of age) slightly, but significantly, attenuated motor disease progression and tended to increase survival time in SOD1G93A mice, suggesting that inhibitory A1R, if anything, are contributing to motor impairment rather than to neuroprotection (Armida et al., 2019). In contrast, the same study showed that the A2AR antagonist, KW-6002, at the same dose and starting day of treatment as in our study, did not affect motor disease progression or survival (Armida et al., 2019). The work by Armida et al. (2019) and our work had clear different objectives, thus distinct parameters were evaluated: disease progression and survival time in their case, synaptic plasticity dysfunction in ours. It is possible that A2AR blockade will only prove beneficial to protect synapses, whereas survival differences will require a more complex therapy. It may also happen that differences in light-protection of KW-6002 may explain, at least in part, negative results in the case of the work by Armida et al. (2019) and positive results in the present work. Altogether, these studies clearly highlight the need of further clarification of the role of each of the adenosine receptor subtypes at different manifestations of the states (Sebastião et al., 2018).

In conclusion, the results herein reported support the idea that ALS should not be considered as a purely motor neuron degenerative disorder, but rather as a multisystemic disorder, that also involves non-motor synapses. Our promising observations in SOD1G93A mice treated with an orally active A2AR antagonist already approved for use in humans, highlight the possibility of a novel approach to fight non-motor symptoms in ALS, eventually frontotemporal dementia (FTD), a common comorbidity of ALS (Strong and Yang, 2011; Wilson et al., 2001; Witgert et al., 2010).

CRediT authorship contribution statement

N. Rei: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. D.M. Rombo: Methodology, Validation, Formal analysis, Investigation. M.F. Ferreira: Formal analysis, Writing - review & editing, Y. Baqi: Resources. C.E. Müller: Resources. J.A. Ribeiro: Writing - review & editing. A.M. Sebastião: Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. S.H. Vaz: Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

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