‘Pre-conditioning’ with Latrepirdine, an AMPK activator, delays ALS progression in SOD1G93A mice

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Running Title: AMPK as a therapeutic target in ALS

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Abbreviations: ALS, Amyotrophic Lateral Sclerosis; AMPK, adenosine 5’-monophosphate-activated protein kinase, SOD1, superoxide dismutase 1; PaGE, paw grip endurance; PND, post-natal day.

Keywords Amyotrophic Lateral Sclerosis, AMPK, bioenergetics, pre-conditioning, spinal cord, motoneuron degeneration, SOD1

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Abstract

Adenosine 5'-monophosphate-activated protein kinase (AMPK) is a master regulator of energy balance. As energy imbalance is documented as a key pathological feature of Amyotrophic Lateral Sclerosis (ALS) we investigated AMPK as a pharmacological target in SOD1\textsuperscript{G93A} mice. We noted a strong activation of AMPK in lumbar spinal cords of SOD1\textsuperscript{G93A} mice. Pharmacological activation of AMPK has shown protective effects in neuronal ‘pre-conditioning’ models. We tested the hypothesis that ‘pre-conditioning’ with a small molecule activator of AMPK, Latrepirdine, exerts beneficial effects on disease progression. SOD1\textsuperscript{G93A} mice (n=24 animals per group; sex- and litter-matched) were treated with Latrepirdine (1 \(\mu\)g/kg, i.p.) or vehicle from postnatal day 70 to 120. Treatment with Latrepirdine increased AMPK activity in primary mouse motor neuron cultures and in SOD1\textsuperscript{G93A} lumbar spinal cords. Mice ‘pre-conditioned’ with Latrepirdine showed a delayed symptom onset and a significant increase in lifespan (p<0.01). Our study suggests that ‘pre-conditioning’ with Latrepirdine may represent a possible therapeutic strategy for individuals harbouring ALS-associated gene mutations who are at risk for developing ALS.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a progressive, neurodegenerative disease that leads to the weakening of motor neurons, paralysis and eventual death. There is no known cure and limited treatment options are currently available. Of the pathological hallmarks of ALS, mitochondrial defects and alterations in energy metabolism routinely correlate with disease progression in patients and in animal models of ALS. In SOD1\textsuperscript{G93A} mice, a mouse model of familial ALS, mutant SOD1 protein accumulates in mitochondria early during disease (Luo \textit{et al.} 2013, Deng \textit{et al.} 2006, Higgins \textit{et al.} 2002). This build up of mutant protein is suggested to impair mitochondrial function (Song \textit{et al.} 2013, Israelson \textit{et al.} 2010, Pasinelli \textit{et al.}
In line with these findings, mitochondria had a metabolic switch from oxidative phosphorylation to glycolysis in transgenic SOD1<sup>G93A</sup> mice, NSC34 cells (motor neuron-like cell line) and fibroblasts transfected with mutant SOD1 compared to controls (Mattiazzi <i>et al.</i> 2002, Richardson <i>et al.</i> 2013, Allen <i>et al.</i> 2013). Glucose utilization and ATP levels have been shown to be significantly reduced in corticospinal and bulbospinal motor tracts and the motor cortex of SOD1<sup>G93A</sup> prior to pathologic changes (Browne <i>et al.</i> 2006). Late-stage SOD1<sup>G93A</sup> mice also display decreased glucose uptake into skeletal muscle (Smittkamp <i>et al.</i> 2014). Furthermore, SOD1<sup>G93A</sup> and SOD1<sup>G86R</sup> mice show a decrease in body weight and fat mass during disease progression, reduced levels of circulating triglycerides, and evidence of increased lipolysis (Dodge <i>et al.</i> 2013, Kim <i>et al.</i> 2011a, Fergani <i>et al.</i> 2007, Dupuis <i>et al.</i> 2004). ALS patients also exhibit higher resting levels of energy expenditure, weight loss, a hypermetabolic phenotype and increased lipolysis (Kasarskis <i>et al.</i> 1996, Desport <i>et al.</i> 2005, Dupuis <i>et al.</i> 2008, Bouteloup <i>et al.</i> 2009, Funalot <i>et al.</i> 2009, Dodge <i>et al.</i> 2013). Hence, there is growing evidence for decreased glucose utilisation and metabolic re-programming in ALS.

Adenosine 5’-monophosphate-activated protein kinase (AMPK) is a major sensor and regulator of energy homeostasis (Hardie <i>et al.</i> 2012). AMPK is a heterotrimeric protein composed of one catalytic subunit (α) and two regulatory subunits (β and γ), and is expressed in most tissues including the CNS (Culmsee <i>et al.</i> 2001). AMPK is activated by phosphorylation of the α subunit at the threonine 172 site when there is a high AMP:ATP ratio (Hawley <i>et al.</i> 1996). AMPK has also been shown to be activated in mutant SOD1-expressing primary mixed spinal cord cultures and in spinal cords of SOD1<sup>G93A</sup> mice at post natal day (PND) 90 (Lim <i>et al.</i> 2012, Perera <i>et al.</i> 2014). When activated in cells, AMPK decreases energy expenditure and increases glucose uptake and utilisation, hence maintaining a bioenergetic homeostasis (Culmsee <i>et al.</i> 2001, Weisova <i>et al.</i> 2009). AMPK also stimulates
fatty acid oxidation and inhibits lipogenesis and triglyceride synthesis (Hardie 2007). Pre-treatment of neurons with AMPK mimetics has been shown to increase the resistance of neurons to subsequent toxic challenges such as Ca\(^{2+}\) overloading (Culmsee et al. 2001, Anilkumar et al. 2013). We have recently also shown that AMPK mediates the preconditioning effects of mild mitochondrial uncoupling and complex inhibition in neurons (Weisova et al. 2012). However, prolonged AMPK activation during severe energetic stress can also lead to pro-apoptotic responses in neurons and can worsen neuronal injury (McCullough et al. 2005). Our laboratory has shown that this effect is mediated via the activation of pro-apoptotic Bcl-2 family proteins (Concannon et al. 2010, Kilbride et al. 2010, Davila et al. 2012). AMPK may therefore act as a ‘master switch’ for neuronal survival decisions (Weisova et al. 2012). However, the role of AMPK in regulating metabolism in ALS and as a new therapeutic target warrants further investigation. Continual AMPK activation may be detrimental to severely stressed neurons (McCullough et al. 2005, Concannon et al. 2010), therefore neuronal ‘pre-conditioning’ rather than continuous treatment with an AMPK activator may represent a novel therapeutic approach for the treatment of subjects at risk of developing ALS. Pre-conditioning is the concept of applying a sub-threshold stress to a tissue which induces tolerance in the case of a more robust stress later (Dirnagl et al. 2003). We have recently identified Latrepirdine as a potent, small molecule activator of AMPK (Weisova et al. 2013). Latrepirdine crosses the blood-brain barrier and is active in the CNS (Wang et al. 2011). It activates AMPK when used in the nanomolar range and has been safely prescribed as a travel sickness remedy and anti-histaminergic agent since the early ‘80s (Weisova et al. 2013, Wang et al. 2011, Kieburtz et al. 2010, Doody et al. 2008, Matveeva et al. 1983). Therefore, here we tested the hypothesis that ‘pre-conditioning’ with Latrepirdine exerts beneficial effects on disease progression in transgenic SOD1\(^{G93A}\) mice.
2. Methods

2.1 Animals

SOD1\textsuperscript{G93A} mice C57B6.Cg-Tg (SOD1G93A) 1Gur/J were purchased from Jackson Laboratory (Bar Harbor, ME, USA). These mice are congenic on the C57Bl6 background and carry a high copy number of the G93A mutant SOD1 transgene. Mice were housed in cages of between 3-5 mice and all mice for this study were age, gender and litter matched according to specific ALS pre-clinical trial guidelines (Ludolph et al. 2010). Mice were housed at constant temperature (22 °C) on a 12 h light/dark cycle (07:00 h on, 19:00 h off), with \textit{ad libitum} food and water available. All experiments were carried out under license (no. B100/4414) from the Department of Health and Children, Ireland, with ethical approval from the Royal College of Surgeons in Ireland Research Ethics Committee (REC625b). (Supplementary table 1 - a record of all animals used in the study, supplementary table 2 – a record of any animals excluded from the study).

2.2 Drugs and ‘Pre-conditioning’ treatment of SOD1\textsuperscript{G93A}tg mice

Latrepirdine (MDV1100) (Medivation, San Francisco, CA, USA) stock was dissolved in ultrapure H\textsubscript{2}O. For \textit{in vitro} use, Latrepirdine was dissolved in culture media and cells were treated at concentrations stated. For \textit{in vivo} use Latrepirdine was diluted in 1X PBS (vehicle). SOD1\textsuperscript{G93A} mice were given Latrepirdine (1 μg/kg/day I.P. injection) or PBS vehicle commencing at PND 70 until PND 120.

2.3 Assessment of lifespan and disease progression \textit{in vivo}

For lifespan and motor function assessment, animals were age-, gender- and litter-matched in accordance with recent ALS guidelines for the generation of preclinical data (Ludolph \textit{et al}. 2010). Tests were performed blind by a single observer, twice weekly with a day interval
between testing. Mice were trained for use of motor function equipment at PND 80. Motor function tests included Rotarod (Stoelting, Illinois, USA), grip strength (Ugo, Basile, Italy) and stride length measurements (Gurney et al. 1994). End stage of ALS disease progression was determined by the extreme weakening of hind limbs and loss of righting reflex within 30 seconds of when mice were placed on their back (Ludolph et al. 2010).

2.4 Assessment of motor neuron survival in vivo

Cryoprotected lumbar spinal cord samples were sectioned (20 µM in thickness) from L1-L5 and Nissl stained with cresyl violet (0.1%). Nissl positive motor neuron cells were counted (according to the pre-determined inclusion criteria – cells must be between 30 µM and 80 µM in diameter, have a dark nucleolus and be a multi-polar in structure) in every third section in the ventral horn region of lumbar spinal cords and motor neuron survival assessed.

2.5 Primary motor neuron cell culture

Primary mixed motor neuron cultures were prepared from E13 C57Bl6 mouse embryos (Sebastia et al. 2009). Briefly, donor animals were terminally anesthetized and embryos removed by hysterectomy. Spinal cords were dissected, the meninges removed and the ventral tissue was incubated in 0.025% trypsin (Sigma, Wicklow, Ireland) for 10 min at 37 °C. The cells were then transferred into Neurobasal medium (supplemented with 2% horse serum (Biosciences, Dublin, Ireland), 2% B27 (Biosciences), 0.1% fungizone (Invitrogen, Dublin, Ireland), GDNF (Promega, Southampton, UK; 2 ng/mL), CNTF (R&D Systems, UK; 1ng/mL), 100 U/mL penicillin and 100 µg/mL streptomycin), with 0.1 mg/ml DNAse 1, (Sigma), and gently dissociated. The cell suspension was centrifuged and the pellet re-suspended in Neurobasal medium. Cells were seeded onto Poly-L-Ornithine/Laminin (Sigma)
coated coverslips at a density of 5x10^4 cells/well in a 24 well plate and maintained in Neurobasal medium at 37 °C and 5% CO₂.

2.6 MTT assay

Cells were plated at 1x10^3 cells/well in a 96 well plate. At DIV 7 cells, were treated with varying concentrations of Latrepirdine (0.01 nM, 0.1 nM, 1 nM, 10 nM, 100 nM) for 24 h. MTT (20 μL; Sigma) was added to each well and incubated for 4 h at 37 °C. The media was carefully removed from each well so as not to remove any crystals. DMSO (200 μL) was added to each well and the plate was placed on an orbital shaker for approx. 10 min to allow the crystals to dissolve. The absorbance was measured at 560 nm absorbance using the Tecan GENios plate reader.

2.7 Immunocytochemistry and confocal imaging

At DIV 7 cells were treated with Latrepirdine [0.1 nM], or Rapamycin [200 nM] or vehicle for 24 h and cathepsin inhibitors pepstatin A and E64D (Calbiochem, Merck Biosciences, Nottingham, UK) for 23 h. Cells were fixed with 3% paraformaldehyde for 12 min at 37 °C. Coverslips were washed three times and permeabilised with 0.1% PBS-tritonX-100. Cells were blocked in 5% goat serum (Sigma), immunostained with primary antibodies; SMI-32 (1 : 500; Abcam, Cambridge, UK) and LC3II (1 : 100; Sigma) and incubated with Alexa Fluor conjugated secondary antibodies (488 nm and 568 nm, 1 : 500; Biosciences) and coverslips again washed thoroughly. Motor neurons were imaged on an LSM 510 Meta Zeiss confocal microscope (Zeiss, Jena, Germany) equipped with a 63× NA 1.4 oil immersion objective. 3D stacks of optical slices were taken with a distance of 0.27 μm and optical slice thickness of 1.1 μm. Processing of immunofluorescence images corresponded to single motoneuron cells (SMI-32) and autophagic vesicles (LC3) in that area and was quantified using CellProfiler (version 2.0, http://www.cellprofiler.org/). Maximum intensity projections were processed
from each stack. From those local background was calculated using local minima (75x75 pixel window for SMI-32 and 15x15 for LC3, respectively) interpolation and subtracted from the raw images. Cells were isolated from background-corrected SMI-32 images using uniform smoothing (15x15 pixel window) followed by Otsu thresholding (two-class, variance ratio minimization). Binary masks corresponding to adjacent vesicles were split using Euclidean distance transform followed by watershedding. Holes in the masks were filled using morphological operations and the masks touching the image edges were rejected from further analysis. Sizes of those masks were measured in pixels and converted to physical areas each representing a cell. The intensity of LC3 was measured and integrated for each cell (SMI-32 image). Vesicles were segmented using Otsu thresholding combined with watershedding on the intensity image, assigned to the corresponding masks and counted on cell-by-cell basis.

2.8 Western Blotting

Samples were homogenised in RIPA buffer (50 nM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with protease inhibitor mixture (1 : 100; Sigma) and phosphatase inhibitors (1 : 100; Sigma). Equal amounts of protein were diluted in Laemmli buffer, separated by SDS-PAGE and transferred to a nitrocellulose membrane using semi-dry transfer apparatus for low molecular weights and wet transfer for high molecular weight proteins. Membranes were blocked using 5% bovine serum albumin (BSA) in TBS-Tween 20 (0.1% ; Sigma) for 1 h at room temperature. Membranes were then incubated with the following antibodies Threonine-172-phospho-AMPK (1 : 1000; Cell Signalling Technology, Boston, MA, USA), AMPK (1 : 1000; Cell Signalling Technology), phospho-ACC (1 : 1000; Cell Signalling Technology), ACC (1 : 1000; Cell Signalling Technology), phospho-mTOR (1 : 1000; Cell Signalling Technology), mTOR (1 : 1000; Cell
Signalling Technology), LC3 (1 : 1000; Sigma), ULK1 (1 : 500; Cell Signalling Technology), p-ULK1(s555) (1 : 1000; Cell Signalling Technology), α-tubulin (1 : 5000; Sigma), β-actin (1 : 5000; Sigma) overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase (HRP) - conjugated secondary antibodies (1 : 5000; Jackson ImmunoResearch) for 1 h at room temperature. Membranes were washed and incubated with enhanced chemiluminescence substrate (Millipore) and imaged using the LAS 3000 Reader (Fujifilm). Densitometric analysis was performed in Image J software on all samples and values were normalised to loading control (α-tubulin / β-actin) and standardized to the corresponding non-tg control.

2.9 Statistical Analysis

Data are presented as mean ± SEM. Motor function tests were analysed by one-way ANOVA and Tukey’s post hoc test. Survival results were analysed by Kaplan-Meier. For Western blot analysis, two-tailed Mann-Whitney U-tests for non-parametric data or two-tailed student t-tests, where applicable. Statistical analysis was performed by PASW statistics version 20 Software (IBM, Dublin, Ireland).

3. Results

3.1 AMPK activation increases throughout disease progression in SOD1G93A mice

The SOD1G93A mouse model is a well-established model of familial ALS and has a relatively low variability in symptom onset and end stage timepoints (Mead et al. 2011). PND 50 is often considered a ‘pre-symptomatic’ timepoint and PND 90 as a symptom onset timepoint when motor dysfunction begins (Mancuso et al. 2011, Mead et al. 2011). At PND 120, symptoms have progressed and there is noticeable hind limb weakness. End stage in SOD1G93A transgenic (tg) mice is determined when animals are unable to ‘right’ themselves
after 30 seconds on their back, known as the righting reflex test (Ludolph et al. 2010). In order to examine AMPK activation across ALS disease progression, we analysed AMPK activation in the lumbar spinal cord homogenates from non-transgenic (non-tg) and tg mice at various timepoints (PND 50, PND 70, PND 90, PND 120 and end stage). Tissue samples were analysed for active, threonine 172-phosphorylated and total AMPK protein levels by Western blot. Acetyl-CoA Carboxylase (ACC) is a downstream target of AMPK which is activated by phosphorylation of serine 79 by AMPK and induces fatty acid oxidation (Park et al. 2002), therefore we also examined phospho- and total ACC levels. At PND 50 and 70, i.e. at ‘pre-symptomatic’ timepoints, there was no significant difference in phospho-AMPK, total AMPK, or the phospho-AMPK/AMPK ratio between non-tg and tg lumbar spinal cords (Fig. 1A). Similarly no difference in the phospho-ACC/ACC ratio was detected. However, at PND 90, AMPK activity was significantly increased in tg lumbar spinal cord samples compared to non-tg samples (Fig. 1B) as indicated by an increase in phospho-AMPK levels relative to β-actin and by the increase in the phospho-AMPK/AMPK ratio. No change in ACC activation was detected at PND 90. Similar results for AMPK activation were observed at PND 120 (Fig. 1C) with increased phospho-AMPK levels relative to β-actin and an elevated phospho-AMPK/AMPK ratio. Interestingly we noted a significant increase in ACC activation levels during disease progression, with significantly elevated levels at PND 120 and end stage. This suggested that the elevated levels of AMPK activation observed in tg mice at PND 90 had a functional effect on the downstream target ACC, with significantly increased ACC activation at PND 120 and end stage. This highlights the sequential activation of ACC following on from AMPK activation. Of note, we also observed a significant decrease in total AMPK levels relative to β-actin at end stage (Fig. 1D). In summary, AMPK activation increased from disease onset, and throughout disease progression in tg lumbar spinal cords, while ACC activation was induced from PND 120 onwards following AMPK activation.
3.2 AMPK activation is associated with autophagy activation during disease progression in SOD1<sup>G93A</sup> mice

Activation of macroautophagy is an event downstream of AMPK activation and can be triggered by inhibition of mTOR (Meley <i>et al.</i> 2006) or via ULK1 phosphorylation (Egan <i>et al.</i> 2011, Kim <i>et al.</i> 2011b). We next determined whether the changes in AMPK activity are also reflected by changes in autophagy. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a ubiquitously expressed protein that is involved in autophagy. When cytoplasmic LC3I binds with phosphatidylethanolamine it forms its associated structure, LC3II. The modification of LC3I to LC3II can be used as an indicator of the autophagy process. We examined LC3I and LC3II protein levels in lumbar spinal cords by Western blot. At PND 50 no difference in LC3II levels were observed between non-tg and tg mice (Fig. 2A). However, at PND 90 there was a significant increase in LC3II levels in SOD1<sup>G93A</sup> lumbar spinal cords (Fig. 2B, Fig. 2E). There was a trend towards increased LC3II levels at PND 120 but this increase was not significant (non-tg 1 ± 0.303 and tg 1.77 ± 0.276, p=0.123). However we observed no notable significant difference in phospho-mTOR, total mTOR or in the phospho-mTOR/mTOR ratio during disease progression in non-tg versus tg mice (Fig. 3 E, 3F, 3G). Additionally, AMPK has been reported to sequentially activate autophagy via the ULK1 pathway. As no change in mTOR levels was detected, next we examined ULK1 levels at PND 90 and PND 120 in SOD1<sup>G93A</sup> mice. Interestingly, we noted a significant increase in p-ULK1 s555 (Egan <i>et al.</i> 2011) and total ULK1 levels at PND 90 (Fig. 4B, Fig. 4C), corresponding with the increase in LC3II levels observed previously at this symptom onset timepoint. In conclusion, we detected an increase in autophagy throughout disease progression, a significant increase in ULK1 signalling at PND 90, but no significant change in mTOR levels indicating the autophagy activation detected was mTOR-independent.
3.3 Latrepirdine activates AMPK and increases LC3II levels in primary motor neurons

We have recently shown that Latrepirdine activates AMPK in the nanomolar concentration range in primary cerebellar granule neurons and cortical neurons (Weisova et al. 2013). To explore whether ‘pre-conditioning’ of SOD1<sup>G93A</sup> mice with Latrepirdine could represent a feasible treatment strategy, we first examined whether Latrepirdine activated AMPK in primary mixed motor neuron cultures. An MTT assay was conducted to test whether Latrepirdine was toxic to primary mixed motor neuron cultures at sub-nanomolar and nanomolar concentrations and it did not affect cell viability (Fig. 5A). Following this, we found that a 24 h treatment with Latrepirdine [0.1 nM] increased phospho-AMPK levels in primary mixed motor neuron cultures evaluated by Western blotting (Fig. 5B), confirming our earlier findings. Primary mixed motor neuron cultures were also treated with Latrepirdine [0.1 nM] for 24 h in the presence of the cathepsin inhibitors (Pepstatin A and E64D) to block the regular recycling of LC3. Cells were then fixed and motor neurons highlighted with an SMI-32 antibody and co-stained with an LC3 antibody. Neurons treated with Latrepirdine showed a significant increase in autophagic flux as indicated by the significant increase in LC3 punctae compared to vehicle-treated controls (Fig. 5G). As a positive control, neurons were treated with the autophagy activator, Rapamycin [200 nM] (Brown et al. 1994). Hence Latrepirdine, at sub-nanomolar concentrations, activated AMPK and had the ability to increase autophagic flux to a similar level as Rapamycin in motor neuron cells. Similarly, by Western blot analysis we examined LC3II levels in primary motor neuron cells treated with Latrepirdine plus inhibitors (Pepstatin A and E64D) and Rapamycin plus inhibitors (Pepstatin A and E64D) (Fig. 5H, Fig. 5I). Similar to results from Fig. 5G, we observed a significant increase in LC3II levels in Latrepirdine plus inhibitor treated cells compared to control.

3.4 Latrepirdine activates AMPK in lumbar spinal cords of SOD1<sup>G93A</sup> mice
Next we administered 1 $\mu$g/kg Latrepirdine or vehicle from PND 70 (pre-symptomatic time point) until PND 120 in SOD1$^{G93A}$ transgenic mice by daily intraperitoneal injections. We chose a dose of 1 $\mu$g/kg which was calculated to yield a tissue concentration of approximately [0.1 nM] in the CNS of the mouse based on previous bioavailability studies after acute dosing (Wang et al. 2011). To explore whether this treatment paradigm activated AMPK levels in vivo, SOD1$^{G93A}$ mice were euthanised at PND 120 following treatment, and lumbar spinal cords collected for Western blot analysis. Fig. 6A and 6B show that Latrepirdine significantly enhanced AMPK activation seen by the increased phospho-AMPK/AMPK ratio in tg mice treated with Latrepirdine compared to tg counterparts treated with vehicle alone. Latrepirdine also tended to increase the phospho-AMPK/β-actin ratio (Vehicle 1 ± 0.300: Latrepirdine 1.8 ± 0.325) however this did not reach the level of statistical significance (p=0.144). LC3II levels were analysed in these samples also to investigate the effect of Latrepirdine treatment on autophagy activation in SOD1$^{G93A}$ transgenic mice. Significantly increased levels of LC3II relative to β-actin were observed in Latrepirdine treated SOD1$^{G93A}$ mice (p=0.045) (Fig. 6C, Fig. 6D).

3.5 Latrepirdine increases life span in SOD1$^{G93A}$ mice

Next we treated SOD1$^{G93A}$ mice from PND 70 to PND 120 with Latrepirdine or vehicle to examine life span, with each treatment group consisting of n=24 litter- and gender-matched animals according to recently published community guidelines (Ludolph et al. 2010). Of note, administration of Latrepirdine to non-tg littermates for 50 days had no effect on mouse viability or behaviour. Kaplan-Meier survival curves representing all animals from the study (males and females combined) showed a significant increase in life span in tg mice treated with Latrepirdine (1 $\mu$g/kg) versus tg mice treated with vehicle (Fig 7A, p=0.002, vehicle – 158 d ± 2, Latrepirdine – 169 d ± 2). Interestingly, when the groups are separated by gender,
males showed a more significant increase in life span (Fig. 7B, p=0.001, vehicle – 158 d ± 2; Latrepirdine – 171 d ± 3), however, in the female group there was a trend towards increased survival but this did not reach the level of statistical significance (Fig. 7C, p=0.107, vehicle - 159 d ± 3, Latrepirdine – 166 d ± 4). From these results, it appears male mice benefit more from Latrepirdine ‘pre-conditioning’ treatment. Of note, as we ascertained that male mice respond better to Latrepirdine treatment compared to females we examined previous samples analysed from Fig. 1-3 with gender groups separated (Suppl. Fig. 1, suppl. Fig. 2.). Interestingly a similar pattern of AMPK, ACC, mTOR and LC3II levels were detected in males alone, females alone and in the combined results which indicate that similar patterns of protein levels are evident independent of gender.

3.6 Latrepirdine delays onset of motor function deficits in SOD1

We also carried out motor function tests on Latrepirdine- and vehicle-treated, male and female mice across disease progression. There was a significant delay in onset of balance and coordination deficits as analysed by rotarod test at PND 145 (p=0.027) and PND 155 (p=0.01) and stride length test at PND 140 (p=0.009). Similarly, a significant delay in onset of forelimb grip strength deficiency was observed at PND 125 (p=0.027) overall in the total groups (data not shown). Similar to the survival data, gender division of the treatment groups revealed that male mice benefitted more from the treatment, and showed a significant improvement in motor function, while female mice showed little or no difference in motor performance abilities. Rotarod analysis in male tg mice treated with Latrepirdine showed a significantly later onset in motor function disabilities at PND 125 (p=0.001), PND 135 (p=0.002) and PND 145 (p=0.003, Fig. 8A). Similarly, male tg mice treated with Latrepirdine showed a significant difference in grip strength at PND 125 when compared to vehicle-treated animals (p=0.029, Fig. 8B). Moreover, there was a significant difference in stride
length in male tg mice treated with Latrepirdine compared to tg animals treated with vehicle at PND 140 (p=0.024, Fig. 8C). Furthermore, a trend towards improved motor neuron survival in males (p=0.094) was detected from Nissl stained motor neuron counts (Fig. 8D). Taken together our results indicate that pre-conditioning with Latrepiridine significantly delays the onset of symptoms in SOD1\(^{G93A}\) transgenic male mice.

4. Discussion

The key finding of this study is that ‘pre-conditioning’ with Latrepirdine increases life span and delays symptom onset in SOD1\(^{G93A}\) mice. We identified increased activation of AMPK in SOD1\(^{G93A}\) mice from symptom onset (PND 90) onwards. Interestingly, we found that Latrepiridine increased AMPK activation and LC3 levels in primary motor neuron cultures, and subsequently designed a ‘pre-conditioning’ treatment model to induce AMPK activation via Latrepirdine treatment in pre-symptomatic/mild symptomatic SOD1\(^{G93A}\) mice. In this pre-clinical study we adhered to the guidelines outlined by Ludolph and colleagues in 2010 including gender matched, litter matched and age matched mice in all aspects of the study (Ludolph et al. 2010). Furthermore, any non-ALS related deaths were excluded from the study (Suppl. table 2.). We found that ‘pre-conditioning’ with Latrepiridine extended life span and improved motor function capabilities in SOD1\(^{G93A}\) mice, and that male mice in particular benefited from this treatment.

AMPK is activated in cases of low ATP availability or a high AMP:ATP ratio to stimulate energy producing mechanisms and inhibit energy consuming pathways in order to restore energy homeostasis (Long and Zierath, 2006). We observed elevated levels of AMPK activation in lumbar spinal cords of SOD1\(^{G93A}\) mice across disease progression. This is in accordance with a previously published study that documented increased AMPK activation at PND 90 in these mice (Lim et al. 2012, Perera et al. 2014). These data may mechanistically
link AMPK activation with previously published bioenergetic abnormalities described in this mouse model (Dodge et al. 2013, Kim et al. 2011a, Fergani et al. 2007, Dupuis et al. 2004). AMPK is able to activate autophagy either through inhibition of mTOR by phosphorylation of TSC2 (Inoki et al. 2003) or raptor (Gwinn et al. 2008), or by direct phosphorylation of ULK1 (Egan et al. 2011, Kim et al. 2011b). Indeed, we observed increased levels of LC3II across disease progression corresponding with previous literature (Morimoto et al. 2007, Li et al. 2008, Tian et al. 2011). Similarly we found elevated ULK1 phosphorylation at PND 90, suggesting that AMPK may signal cytoprotective mechanisms such as the clearance of protein aggregates (Hetz et al. 2009, Crippa et al. 2010). In contrast, we found no evidence of increased mTOR phosphorylation with Latrepirdine treatment suggesting that any increase in the autophagy process does not occur via mTOR activation. Interestingly, treatment of SOD1\textsuperscript{G93A} mice with the mTOR inhibitor, Rapamycin, failed to show beneficial effects in a previous study (Zhang et al. 2011) while trehalose treatment, which induced mTOR-independent autophagy, delayed disease progression in SOD1\textsuperscript{G93A} mice (Castillo et al. 2013).

The right balance of AMPK activation is pinnacle to cell survival as prolonged activation of AMPK can lead to cell death (Weisova et al. 2012). Transient AMPK activation can have pro-survival effects in neurons which are partially mediated by increased GLUT 3 translocation to the cell membrane and increased glucose uptake and utilization (Weisova et al. 2009, Culmsee et al. 2001, Gundewar et al. 2009). On the contrary, increased AMPK activation has been associated with excitotoxicity, stroke-induced injury \textit{in vivo}, and neurodegeneration in Huntington’s disease (McCullough et al. 2005, Concannon et al. 2010, Mochel et al. 2012). Nuclear translocation of AMPK and phosphorylation of the pro-apoptotic transcription factor FOXO3A by AMPK have been linked to the upregulation of pro-apoptotic pathways in response to prolonged or uncompensated AMPK activation (Concannon et al. 2010, Davila et al. 2012, Mochel et al. 2012). These studies suggest that
the correct level of AMPK activation is crucial to maintain neuronal survival, but importantly also suggested that treatment with AMPK activators may be detrimental during late stage of disease progression.

We therefore successfully designed a treatment regimen where Latrepirdine was administered to SOD1\textsuperscript{G93A} mice during the ‘pre-symptomatic’ and mid symptomatic periods only. Using this treatment paradigm, Latrepirdine improved survival and delayed disease onset and progression in SOD1\textsuperscript{G93A} mice. A previous preliminary report showed no benefit of Latrepirdine treatment in SOD1\textsuperscript{G93A} mice, however, animals were treated with (20 mg/kg) per day from symptom onset up until end stage (Tesla \textit{et al.} 2012). Other AMPK modulating agents have also previously been suggested to be protective for motoneurons or improve disease progression in ALS and other neurodegenerative disorders. Resveratrol leads to AMPK activation and regulates energy expenditure via downstream activation of SIRT1 (a NAD+-dependent deacetylase) (Tennen \textit{et al.} 2012, Canto \textit{et al.} 2009). Treatment with Resveratrol was found to attenuate neurotoxicity in primary neurons transfected with mutant SOD1\textsuperscript{G93A} and mutant Huntington mice (HdhQ111 knock-in mice) (Kim \textit{et al.} 2007, Parker \textit{et al.} 2005). Resveratrol treatment has also been shown to be beneficial in a model of Alzheimer’s disease (SAMP8 mice) (Porquet \textit{et al.} 2013). \textit{In vivo} treatment with Resveratrol (160 mg/kg) from a pre-symptomatic timepoint (8 weeks old) significantly improved upper and lower motor neuron survival and increased life span in SOD1\textsuperscript{G93A} mice (Mancuso \textit{et al.} 2014). However, Resveratrol has a very limited safety profile available and has not been used in many clinical trials to date (Patel \textit{et al.} 2011, Smoliga \textit{et al.} 2011). Interestingly, Riluzole, the only FDA approved drug for the treatment of ALS, has recently been suggested to be an AMPK activator in the motor neuron-like cell line, NSC-34 cells and L6 myotubes (Daniel \textit{et al.} 2013), but further \textit{in vivo} studies are warranted to explore the role of AMPK activation and its potential neuroprotective activities. Treatment with the AMPK activating compound
Metformin (0.5, 2, 5 mg/ml) in drinking water from PND 35 onwards did not increase life span or have any effect on disease progression in SOD1^{G93A} mice (Kaneb et al. 2011). However, metformin is primarily absorbed in the small intestine and expended in the liver which may explain why it was not favourable as a neurodegeneration drug treatment (Chen et al. 2013).

Dietary intervention for the treatment of ALS has also been shown to be successful. A prophylactic treatment with a high ketogenic diet in SOD1 mice increased energy production in mitochondria and showed a significant increase in motor neuron survival and decreased weight loss (Zhao et al. 2006). Moreover, caprylic triglyceride attenuated ALS motor function deficits in SOD1^{G93A} mice by significantly enhancing the mitochondrial oxygen uptake (Zhao et al. 2012). A high-energy diet increased survival in SOD1^{G86R} mice by 20% (Depuis et al. 2004). Hypercaloric dietary supplementation (high carbohydrate) has also been shown to be successful in ALS patients in a randomised, double-blind, placebo-controlled phase 2 trial (Wills et al. 2014). Dietary restriction (DR), in contrast, although activating AMPK, was mainly unsuccessful in extending life span in SOD1^{G93A} mice (Patel et al. 2010, Hamadeh et al. 2005, Pedersen and Mattson, 1999). Hence AMPK activation via dietary restriction may not be a feasible approach for the treatment of ALS as the increased ATP energy level demand may not be attainable with reduced calories.

Interestingly, while ‘pre-conditioning’ with Latrepirdine had little or no effect on female survival the treatment had a profound effect on survival in the males, indicating that the effect could be hormonally linked. Oestrogen is a potent neuroprotective, anti-oxidant which may have interfered with Latrepirdine treatment rendering the drug ineffective in the female mice (Behl 2002). Alternatively, bioenergetic stress may be more pronounced in male SOD1^{G93A} mice and hence this treatment may provide the most beneficial results in males.
Our results suggest that ‘pre-conditioning’ treatment with Latrepirdine would have beneficial effects in delaying symptom onset in ALS patients. But, are pre-conditioning treatments feasible in the future? Genetic testing for mutations associated with ALS to identify subjects at risk is becoming increasingly important. For example, genetic screening for C9orf72 repeat expansions identified 51.6% of cases of fALS and 9.6% of sALS cases (Debray et al. 2013). Therefore, with the increased identification of genes conferring significant risks for the development of ALS or other neurodegenerative disorders, the use of ‘pre-conditioning’ treatment paradigms may become tangible in the near future.

In conclusion, we here provide evidence that ‘pre-conditioning’ with an AMPK activator exerts protective effects in SOD1\textsuperscript{G93A} mice, and identify Latrepirdine as a possible therapeutic intervention for the treatment of subjects at risk of developing ALS.

**Figure Legends**

**Figure 1. Increased AMPK activation throughout ALS disease progression in SOD1\textsuperscript{G93A} mice**

Western blots of phospho- and total AMPK and ACC protein levels at A) PND 50, non-tg n≥5 and tg n≥9 B) PND 90, non-tg n≥3 and tg n≥5 C) PND 120, non-tg n≥3 and tg n≥5. D) End stage, non-tg n≥5 and tg n≥9 E) Quantification of phospho-AMPK levels relative to β-actin showing a significant increase in phospho-AMPK phosphorylation at PND 90 (*p=0.0021) and PND 120 (*p=0.0055). F) Quantification of AMPK levels relative to β-actin across disease progression. A significant decrease in total AMPK levels was identified at end stage (*p=0.0056). G) Quantification of the ratio of phospho-AMPK levels relative to total AMPK levels where a significant increase in the ratio was observed at PND 90 (*p=0.0177) and PND 120 (*p=0.0112). H) Quantification of phospho-ACC levels relative to β-actin across disease progression showing a significant increase in phospho-ACC at PND 120...
(*p=0.0357) and end stage (*p=0.0028). I) Quantification of total ACC levels relative to β-actin across disease progression. A significant decrease in ACC levels was identified at PND 50 (*p=0.0032). J) Quantification of the ratio of phospho-ACC relative to total ACC where a significant increase in the ratio was observed at end stage (*p=0.0112). Data are presented as mean ± SEM and statistically analysed by Mann-Whitney U tests. Values are normalised to loading control and tg values are displayed relative to non-tg counterparts.

Figure 2. AMPK activation is associated with autophagy activation during disease progression in SOD1<sup>G93A</sup> mice

Western blots of LC3I and LC3II protein levels at A) PND 50, non-tg n≥5 and tg n≥9 B) PND 90 non-tg n≥3 and tg n≥5 C) PND 120 non-tg n≥3 and tg n≥5 D) End stage non-tg n≥5 and tg n≥9 E) Quantification of LC3II expression levels relative to β-actin showing a significant increase at PND 90 (*p=0.0357). Data are presented as mean ± SEM and statistically analysed by Mann-Whitney U tests. Values are normalised to loading control and tg values are displayed relative to non-tg counterparts.

Figure 3. No change in mTOR activity throughout ALS disease progression in SOD1<sup>G93A</sup> mice

Western blots of phospho-mTOR and total mTOR protein levels at A) PND 50, non-tg n≥5 and tg n≥9 B) PND 90, non-tg n≥3 and tg n≥5 C) PND 120, non-tg n≥3 and tg n≥5 D) End stage, n≥5 and tg n≥9 E) Quantification of phospho-mTOR levels relative to β-actin. F) Quantification of total mTOR levels relative to β-actin. G) Quantification of the ratio of phospho-mTOR levels relative to total mTOR levels shows no significant differences across disease progression. Data are presented as mean ± SEM and statistically analysed by Mann-
Whitney U tests. Values are normalised to loading control and tg values are displayed relative to non-tg counterparts.

Figure 4. Increased ULK1 activation is associated with autophagy activation and AMPK activation during disease progression in SOD1G93A mice

Western blots of phospho-ULK1 s555 (Egan et al. 2011) and total ULK1 protein levels at A) PND 90, non-tg n≥3 and tg n≥3. B) Quantification of phospho-ULK1 levels relative to β-actin showing a significant increase at PND 90 (*p=0.0053). C) Quantification of total ULK1 levels relative to β-actin where a significant increase in ULK levels was observed (*p=0.0027). D) Quantification of the ratio of phospho-ULK1 levels relative to total ULK1 levels showed no significant differences across disease progression. Western blots of phospho-ULK1 and total ULK1 protein levels at E) PND 120, non-tg n≥4 and tg n≥4. F) Quantification of phospho-ULK1 levels relative to β-actin. G) Quantification of total ULK1 levels relative to β-actin. H) Quantification of the ratio of phospho-ULK1 levels relative to total ULK1 levels. No significant difference in ULK1 signalling was recorded at PND 120. Data are presented as mean ± SEM and statistically analysed by unpaired Student’s t-tests. Values are normalised to loading control and tg values are displayed relative to non-tg counterparts.

Figure 5. Latrepirdine increased AMPK phosphorylation and LC3II levels in primary mixed motoneuron cultures

A) MTT cell viability assay in primary mixed motoneurons treated with varying concentrations of Latrepirdine (0.01, 0.1, 1, 10, 100 nM) for 24 h, n=3, data displayed as mean ± SEM B) Western blot of phospho-AMPK and AMPK protein levels in primary mixed motoneuron cultures treated with varying concentrations of Latrepirdine for 24 h, n=3 C) Quantification of phospho-AMPK protein levels relative to β-actin. Latrepirdine at 0.1 nM
concentration significantly increased AMPK phosphorylation (*p=0.053) D) Quantification of total AMPK levels relative to β-actin. E) Quantification of phospho-AMPK/AMPK ratio where a significant increase was observed at the 0.1 nM concentration (*p=0.036). F) Fluorescent maximal intensity 3D images of primary motor neuron cells (DIV 7) marked with SMI-32 (green) and co-stained with LC3 (red) after vehicle, Latrepirdine [0.1 nM] or Rapamycin [200 nM] treatment for 24 h (plus pepstatin A and E64D). G) LC3 punctae counts relative to motor neuron cell area (represented per 100 μM²). Latrepirdine significantly increased LC3 punctae accumulation in motor neurons (*p<0.001) and Rapamycin significantly increase LC3 punctae (*p<0.001). Data are presented as mean ± SEM and analysed by one-way ANOVA with Tukey post hoc test. Scale bar = 10 μM, vehicle n=15 cells, Latrepirdine n=14 cells and Rapamycin n=19 cells. H) Western blot of LCI and LC3II levels in primary mixed motor neuron cultures treated with vehicle, Latrepirdine [0.1 nM] or Rapamycin [200 nM] for 24 h (with or without pepstatin A and E64D). I) Quantification of LC3II levels relative to β-actin in primary motor neuron cultures treated with Latrepirdine plus inhibitors (n=3) and Rapamycin plus inhibitors (n=2). A significant increase in LC3II levels was observed in cells treated with Latrepirdine plus inhibitors (*p<0.05) compared to control.

**Figure 6. ‘Pre-conditioning’ with Latrepirdine increased AMPK levels in SOD1<sup>G93A</sup> mouse spinal cords**

A) Western blot of phospho-AMPK and AMPK levels in SOD1<sup>G93A</sup>tg lumbar spinal cords (at PND 120) treated with vehicle or Latrepirdine (1 μg/kg) from PND 70 - PND 120 (vehicle treated n=3, Latrepirdine treated n=4) B) Quantification of phospho-AMPK/AMPK ratio relative to β-actin where there was a significant increase in the ratio between tg samples treated with vehicle verses tg samples treated with Latrepirdine (*p=0.013). C) Western blot
of LC3I and LC3II levels in SOD1<sup>G93A</sup>tg lumbar spinal cords (at PND 120) treated with vehicle or Latrepirdine (1 µg/kg) from PND 70 - PND 120 (vehicle treated n=3, Latrepirdine treated n=4) D) Quantification of LC3II levels relative to β-actin with a significant increase between tg samples treated with vehicle verses tg samples treated with Latrepirdine (*p=0.045).

Figure 7. ‘Pre-conditioning’ treatment with Latrepirdine significantly increased life span in SOD1<sup>G93A</sup> mice

Kaplan–Meier analysis of probability of survival of vehicle treated tg groups compared to Latrepirdine treated tg groups with A) total mice (male and female), vehicle n=22 and Latrepirdine [1 µg/kg] n=24. There was a significant increase in life span in total Latrepirdine treated tg group compared to vehicle (p=0.002) where vehicle treated mice lived for an average of 157.75 d (± 2) and Latrepirdine treated mice lived for an average of 168.83 d (± 2) B) male mice alone, vehicle n=11 and Latrepirdine [1 µg/kg] n=11, where Latrepirdine treated tg group compared to vehicle (p=0.001), vehicle treated lived for an average of 158.27 d (± 2) and Latrepirdine treated mice lived for an average of 171 d (± 3) C) female mice alone, vehicle n=11 and Latrepirdine [1 µg/kg] n=13. Although there was a trend towards survival in the female group it did not reach the level of statistical significance (p=0.107, average vehicle 159.18 d (± 3) and average Latrepirdine 165.9 d (± 4).

Figure 8. Latrepirdine treatment delays onset of motor function impairment in male mice

A) Rotarod analysis in males across disease progression in treated SOD1<sup>G93A</sup> mice. There was a significant delay in the onset of motor function deficits in rotarod performance in SOD1<sup>G93A</sup> tg males treated with Latrepirdine versus male tg treated with vehicle (*p=0.001, **p=0.002, ***p=0.003). B) Forelimb grip strength in males analysis across disease
progression where a significant increase in strength was observed at approx. PND 125 (*p=0.007). C) Stride length analysis in males showed a significant improvement in tg males treated with Latrepirdine (*p=0.024) compared to tg mice treated with vehicle. There was no significant difference seen in female groups treated with vehicle or Latrepirdine [1 µg/kg] for rotarod, grip strength or stride length tests. D) Motor neuron survival analysis at PND 120 in tg males. A trend towards increased motor neuron survival was detected in tg males treated with Latrepirdine (p=0.094) compared to tg treated with vehicle (tg vehicle treated n=6, tg Latrepirdine treated n=7).

Acknowledgements

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Authorship credits

K.S.C and J.H.M.P. designed research; K.S.C and M.R.M performed research; K.S.C., M.C.H. and J.H.M.P analysed data; K.S.C., M.C.H and J.H.M.P wrote the article.

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Figure 1:

A

55kDa

250kDa

36kDa

PND 50

B

non-tg  SOD1

55kDa

250kDa

36kDa

PND 90

C

non-tg  SOD1

55kDa

250kDa

36kDa

PND 120

D

non-tg  SOD1

55kDa

250kDa

36kDa

END

p-AMPK

p-ACC

β-Actin

AMPK

ACC

β-Actin

E

p-AMPK

relative to β-Actin

F

AMPK

relative to β-Actin

G

p-AMPK/AMPK

relative to β-Actin

H

p-ACC

relative to β-Actin

I

ACC

relative to β-Actin

J

p-ACC/ACC

relative to β-Actin

Figure 2:

A

28kDa

11kDa

36kDa

PND 50

B

non-tg  SOD1

28kDa

11kDa

36kDa

PND 90

C

non-tg  SOD1

28kDa

11kDa

36kDa

PND 120

D

non-tg  SOD1

28kDa

11kDa

36kDa

END

LC3 I

LC3 II

β-Actin

E

LC3 II

relative to β-Actin

0.5

1.0

1.5

2.0

2.5

3.0

50

90

120

END
Figure 5:
Figure 6:

A

B

C

D
Figure 7:

A

B

C

Kaplan–Meyer analysis

Vehicle

Latrepirdine [1 μg/kg]

Survival probability (%)

Age (days)

Survival probability males (%)
Figure 8:

A

B

C

D

Strides length (mm)

Average no. of motoneurons per ventral horn
Supplementary Figure 1:
Supplementary Figure 2:
Table 1:
### Table 1 - Age- litter- and gender-matched mice used in the study

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### Table 2 - Mice excluded from the study

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<td>Veh</td>
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</tbody>
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