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7,8-Dihydroxyflavone improves motor performance and enhances lower motor neuronal survival in a mouse model of amyotrophic lateral sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is an enigmatic neurodegenerative disorder without any effective treatment characterized by loss of motor neurons (MNs) that results in rapidly progressive motor weakness and early death due to respiratory failure. Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family known to play a prominent role in the differentiation and survival of MNs. The flavonoid 7,8-dihydroxyflavone (7,8-DHF) is a potent and selective small molecule tyrosine kinase receptor B (TrkB) agonist that mimics the effects of BDNF. In the present study, we evaluated the neuroprotective effects of 7,8-DHF in a transgenic ALS mouse model (SOD1^{G93A}). We found that chronic administration of 7,8-DHF significantly improved motor deficits, and preserved spinal MNs count and dendritic spines in SOD1^{G93A} mice. These data suggest that 7,8-DHF should be considered as a potential therapy for ALS and the other motor neuron diseases.

Keywords

Amyotrophic lateral sclerosis; 7, 8-dihydroxyflavone; motor neurons; BDNF; TrkB

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Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive, fatal, and untreatable neurological disease characterized by muscle weakness, atrophy and spasticity, typically leading to paralysis and death within 3–5 years after symptom onset [1]. Pathologically, ALS is primarily characterized by degeneration and death of upper and lower motor neurons (MNs) in the cerebral cortex, brainstem, and spinal cord [1,2]. Most cases of ALS are sporadic (sALS) and of unknown etiology but approximately 5–10% of patients have a clear family history (fALS), typically as an autosomal dominant trait [3]. The clinical course of the disease is highly variable suggesting that the selective vulnerability of MNs likely arises from a combination of factors, including protein misfolding, mitochondria dysfunction, oxidative damage, defective axonal transport, excitotoxicity, insufficient growth factor signaling and inflammation [4]. At least 14 genes and loci have been identified to be mutated in ALS [5]. Mutations in the superoxide dismutase 1 (SOD1) gene accounts for 20% of fALS and apparently for 5% of sALS [6]. The role of SOD1 in ALS is not completely understood, but it is thought that a toxic gain of function rather than a loss of dismutase activity are responsible for the motor neuron loss [7].

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, is known to support motor neuron differentiation and survival [8]. It exerts its effects through two transmembrane receptors: the p75 neurotrophin receptor (p75^{NTR}) and the tyrosine kinase receptor B (TrkB), the primary receptor for BDNF [9]. BDNF is of particular therapeutic interest because of its neurotropic actions on neuronal populations involved in several disorders such as amyotrophic lateral sclerosis [10], Parkinson's disease, and Alzheimer's disease [11]. However, clinical trials using recombinant BDNF have been disappointingly negative, presumably because of poor delivery to the central nervous system (CNS) and short half-life.

Recent screening of a chemical library has identified a flavone derivative 7,8-dihydroxyflavone (7,8-DHF) as the first small-molecule compound that crosses the blood brain barrier (BBB) and binds with high affinity and specificity to the BDNF receptor TrkB (dissociation constant $K_d = 320\text{nM}$) and activates its downstream signaling cascade [12]. Flavonoids, present in fruits and vegetables, have been shown to exert diverse biological actions including neuroprotective, anti-oxidant and anti-apoptotic properties. 7,8-DHF not only a neuroprotective agent but may also regulate neuromuscular transmission [13]. Neurotrophin signaling at the neuromuscular junction modulates cholinergic transmission [13,14,15] and BDNF potentiates neurotransmitter release in both developing neuromuscular synapses in culture [14,15,16], and in adult rat neuromuscular junctions [13]. 7,8-DHF appears to have a number of beneficial effects in different model systems. For example, it blocks caspase-3 and promotes neurogenesis potentiating antidepressant drug actions [17], improves learning and spatial memory in stressed mice through effects on the amygdala [18], increases neuronal nuclei size, enhances locomotor activity and improves breath instability in Rett syndrome mice [19], and reduces brain atrophy and improves survival and motor deficits in a mouse model of Huntington disease [20]. The effect of 7,8-DHF on ALS is not known. In the present study, we investigated the therapeutic effects of 7,8-DHF on

motor performance, spine density and lumbar spinal motor neuron count in a transgenic mouse model of ALS (SOD1^{G93A}).

Materials and Methods

Transgenic mice, breeding and genotyping

Transgenic mice with the G93A human SOD1 mutation (B6SJLTgN(SOD1^{G93A})1 Gur; Jackson Laboratories, Bar Harbor, ME, USA) were bred with female B6SJL mice (Jackson Laboratories). Only male transgenic and wild type (WT) mice were used in the present study. Offspring male were genotyped by PCR on DNA extracted from tail clippings. At weaning (around 30 days of age), male transgenic SOD1^{G93A} mice from the same “F” generation were randomly distributed in 2 different experimental groups: 7,8-dihydroxyflavone (7,8-DHF) treated and untreated (saline injected) ALS groups (n=10 for each group). A group of wild-type (WT) littermate mice were also used in this study. All animal experiments were carried in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local animal care committee.

Treatment protocol

Starting at one month of age and until 105 days of age, SOD1^{G93A} transgenic and non-transgenic WT mice were injected with 7,8-DHF (Tocris Bioscience, Ellisville, MO) (5mg/kg, i.p./3 days a week - dilution of DHF: 5mg/ml- for each 20 g mouse approximately 0.02 ml) or saline (DHF's diluent).

Body weight and motor performance test

Starting at one month of age, body weight and motor performance were monitored twice a week, at the same time of the day. Motor performance test was done on a rotarod apparatus (Columbus Instrument, Columbus, OH, USA) after 2 days of training to get acquainted with the apparatus. The motor performance test consisted in 3 consecutive trials of 60 s each on the rotarod at 11 rpm. The time until mice fell from the rod was recorded and the best of the three trials was used as the measure of competence on the task for that day.

Tissue preparation for post-mortem studies

Mice were euthanized at 105 days of age by CO₂ suffocation. The lumbar section of the spinal cord was dissected out using the anatomical features of the cords. The lumbar section was and divided into two parts: the more proximal part to be used for cresyl violet (CV) staining and the more distal for Golgi staining. For CV staining, tissue was fixed in cold Periodate-Lysine-Paraformaldehyde (PLP) solution at 4°C and cryoprotected in 10 and 20% glycerol/2% DMSO solutions. Tissue was serially sectioned at 60µm thickness on a freezing sliding microtome and saved in PBS/2 mM sodium azide at 4°C. Free floating sections were stained with cresyl violet to quantify the number of MNs. Golgi staining was performed using the FD Rapid GolgiStain Kit (FD NeuroTechnologies) following the manufacturer's instructions. Stained tissue was serially sectioned at 100µm thickness using a cryostat microtome and used to quantify the spine density.

Quantification of motor neurons

Stereological methods were employed to quantify the number of motor neurons (MNs) in the ventral horn of lumbar cord using an Optical Fractionator probe. A computer software package, StereoInvestigator (MicroBright-Field, Colchester, VT), interfaced with a Nikon Eclipse 80i microscope equipped with Ludl motorized stage, Optronics Microfire color digital camera with 1600×1200 resolution and Heidenheim Z axis encoder was used to collect and analyze the stereological data. Using the StereoInvestigator's optical fractionator probe, the total number of MNs was estimated from coded slides. To count each case we used 4 section, each 1080 μm apart (one every 16 sections). The area of interest, the ventral horn defined as the anterior subdivision of the gray matter to the middle of the central canal, was traced in the CV stained sections at $\times 4$ magnification. The size of the counting frame was $75 \times 75 \mu\text{m}$ and the area counted (XY) = $5625 \mu\text{m}^2$. Cell counting was performed using a $\times 60$ N.A 1.4 oil objective. Neurons were only counted if their diameter was $15 \mu\text{m}$ or larger and only if the neuron nucleolus was inside the counting frame and the neuron was not touching the excluding borders. The computer cursor was set at $15 \mu\text{m}$ long for easy detection of cells that meets the $15 \mu\text{m}$ diameter criterion. Neurons were marked with two different marker depending on size, $30 \mu\text{m}$ diameter or larger and smaller than $30 \mu\text{m}$ but larger than $15 \mu\text{m}$. These MNs referred in text as small and large MNs, respectively.

Spine Density

The density of dendritic spines was quantified by counting spines in $25 \mu\text{m}$ length of secondary branches of dendrite on selected motor neurons (Figure 3A). Spine counting was performed using a $\times 100$ N.A 1.4 oil objective. Four sections with 3 section interval, each separated by $1200 \mu\text{m}$ from the superior to the inferior lumbar part of spinal cord, were used to count each case. We analyzed spine density on ventral horn motor neuron using the following criteria: Golgi-stained neurons having dendrites and spines that were completely impregnated, appearing as a continuous length; neurons with at least two dendrite extended into the origin of the cell body; neurons with a soma larger than $15 \mu\text{m}$ were counted. Spine density is defined as the number of spines per micrometer of dendrite length. Images were captured with a Nikon Eclipse 80i microscope with a computer software package, StereoInvestigator (MicroBrightField, Colchester, VT). The values are calculated by dividing the total spine numbers to the length of branches ($25 \mu\text{m}$)

Statistical analysis

Data are presented as the means and standard errors (mean $\pm$ SEM) for each group, and $p < 0.05$ was considered significant. Data from rotarod test and body weight were analyzed using the repeated measures multi-way ANOVA, followed by the Dunns test for posthoc comparisons. Statistical evaluation of the neuronal count and spine density were performed using one-way ANOVA, followed by Tukey's HSD test for multiple comparisons.

Results

Body weight of the untreated SOD1^{G93A} mice was significantly lower than those of WT mice. Difference in body weight were first observed at 60 days of age and persisted until the

final day (105 days). Treatment with 7, 8-DHF did not prevent loss of body weight in the transgenic mice (Figure 1A).

Similarly, motor performance deficits were first detected after 60 days age and persisted until death in WT and saline treated SOD1^{G93A} mice. 7,8-DHF treatment significantly improved motor performance of SOD1^{G93A} mice ($p<0.001$, Figure 1B).

Motor neurons counts in saline treated SOD1^{G93A} mice were significantly reduced compared to WT groups ($p<0.001$, Figure 2). 7,8-DHF treatment prevented MN loss (Figure 2E). 7,8-DHF treatment preserved MN counts of small but not large MNs (Figure 2 F, G).

Dendritic spine density of untreated SOD1^{G93A} mice was reduced compared to the WT controls ($p<0.001$). Dendritic spine density of MNs in 7,8-DHF treated SOD1^{G93A} mice, however, were significantly higher than saline treated transgenic mice and did not differ from that of WT mice ($p<0.001$, Figure 3).

Discussion

This study was designed to determine the prophylactic effect of the long-term treatment with 7,8-DHF, a TrkB agonist, in SOD1^{G93A} mice. Treatment started at 1 month of age when disease symptoms are not yet apparent and ended at 105 days of age, at an advanced stage of the disease but before the terminal-stage to avoid that the rapid progression of the pathological process in its final phase could masked the potential beneficial effect of 7,8-DHF. Because there are no studies on the effect of 7,8-DHF on ALS animal models we chose the dose of 5mg/kg and the i.p. route based on previous studies in mouse models of stroke, PD, and AD [12,21]. In those studies mice were treated daily with 5mg/kg i.p. for 10 and 14 days. Because the overexpression of BDNF has been shown to have detrimental effects in the brain [22] and our treatment period was much longer than those previously reported we decided to treat mice 3 times a week only. Moreover, this intermittent treatment paralleled the schedule of our previous work in which SOD1^{G93A} mice were treated for 30 min 3 days a week with moderate level exercise, which is known to increase BDNF/TrkB signaling [23]. 7,8-DHF treatment significantly preserved motor performance and lumbar spinal motor neurons in ALS mice without reversing the weight loss that characterizes the disease. Additionally, this study also reveals the differential effect of 7,8-DHF treatment on lumbar spinal motor neuron subtypes at the dosage used.

A recent report has been published [24] describing for the first time the in vivo pharmacokinetic properties of 7,8-DHF and its metabolites. In that study, mice were treated by oral gavage with 50 mg/kg, a dosage that is 10-fold the widely used therapeutic dose. The concentration of 7,8-DHF in the plasma peaked at 10 min (70 ng/ml) and was still detectable at 8 h (5 ng/ml) ($T_{1/2}$ = 134min). In the brain 7,8-DHF also peaked at 10 min (52 ng/g), decreased to 18 ng/g at 30 min, and remained relatively stable until 240 min (7 ng/g). The orally administered 7,8-DHF was mainly metabolized to glucuronidated and O-methylated 7,8-DHF. The parent drug and the methylated metabolite penetrate the BBB, where they both activated TrkB receptors. Since the concentration of 7,8-DHF in the brain was approximately 30-fold higher than that of the methylated form it was expect that the main

contribution to TrkB activation came from 7,8-DHF itself. In the present study we used different dosage and route of administration and the kinetics of 7,8-DHF may differ from those described. Because of the low $T_{1/2}$ and low toxicity of 7,8-DHF, increasing the treatment frequency used in our studies may result in broader and stronger beneficial effects.

Spinal MNs are a highly diverse group in terms of their morphology, connectivity, and functional properties and differ significantly in their response to disease. Motor neurons can be classified in “small” ($< 30\mu\text{m}$ in soma diameter) or “large” ($30\mu\text{m}$ or greater in diameter) [25]. MNs are categorized as alpha (α), beta (β) or gamma (γ) MNs. β - and γ -MNs are the smallest MNs and innervate intrafusal fibers of the muscle spindle and regulate the muscle tonus [26]. α -MNs are larger and innervate extrafusal skeletal muscle cells that drive muscle contraction [25]. In this study, 7,8-DHF treatment of $\text{SOD1}^{\text{G93A}}$ mice resulted in the preferential preservation of small MNs. One possible explanation for our observations is shrinkage but persistence of large MNs that would otherwise die without 7,8-DHF treatment. The normal morphology of the small MNs would speak against this possibility. If the persistent neurons are γ -MNs they could improve motor performance indirectly by priming extrafusal fibers innervated by large MNs. The preserved neurons may be small α -MNs that have higher input resistance and require less synaptic activation to initiate action potentials. During muscle contraction small α -MNs reach threshold potential earlier than large MNs, this is known as “size principle” [25,26]. Moreover, while large α -MNs are mainly employed in short-lasting bouts of forceful contraction (e.g. running, jumping), called fast α -MNs, on the other hand, small α -MNs, dominantly get involved in long-term tonic phase of contraction, such as postural tasks, called slow α -MNs [27]. Histological studies in postmortem tissue from ALS patients show γ -MNs deprivation alongside loss of α -MNs [28]. However, a study using $\text{SOD1}^{\text{G93A}}$ transgenic mice showed that α -MNs are more vulnerable than γ -MNs and concluded that α -MNs loss is primarily seen in this model [29]. Our study showing increased number of small MNs but not large MNs in treated mice suggests that 7,8-DHF stimulates the survival of γ -MNs and small α -MNs that results in improved motor performance on the rotarod. A study has shown that in WT rats the expression of TrkB does not correlate with motor neuronal size [30] however, no studies have been done in $\text{SOD1}^{\text{G93A}}$ mice to determine how the disease process may affect the level of TrkB. Growing evidence suggests that neuronal activity enhances BDNF signaling by increasing the cell-surface expression of TrkB and promoting TrkB endocytosis, a signaling event important for many long-term BDNF functions [31]. TrkB levels could be influenced under a variety of pathological conditions known to alter neuronal activity. Since smaller MNs are more easily activated than larger motor units we speculate that in $\text{SOD1}^{\text{G93A}}$ mice γ -MNs and small α -MNs have more TrkB receptors than large α -MNs and therefore they are more responsive to 7,8-DHF treatment.

BDNF is one of the most potent modulators of synaptic transmission, plasticity, and morphology [32,33]. It increases dendritic spine density in a variety of CNS neurons [34,35]. Dendritic spines increase the connectivity of the dendrites and regulate input-specific synaptic plasticity [36]. They have also an electrical role by filtering synaptic potentials and isolating inputs from each other [37]. We found that 7,8-DHF increases MNs dendritic spine density in $\text{SOD1}^{\text{G93A}}$ transgenic mice. It would be interesting to know whether the effect of 7,8-DHF on dendritic spine density correlate with neuronal size.

Conclusion

Chronic 7,8-DHF treatment from 30 days to 105 days improved motor deficits with a differential effect on small-MNs in the ventral horn of lumbar spinal cord of SOD1^{G93A} mice. Spine density was also increased in treated mice. 7,8-DHF could be an important therapeutic tool for ALS and other neurodegenerative disorders. Studies at different time points (95 days – when the symptoms start and 120 days -terminal stage) are underway to further characterize the effects of 7,8-DHF on SOD1^{G93A} mice.

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Highlights

We tested the effect of 7,8-DHF on lower motor neurons in ALS mice.

Chronic administration of 7,8-DHF improved motor deficits in ALS mice.

7,8-DHF preserved spinal MNs and dendritic spines in ALS mice.

7,8-DHF had a differential effect on small-MNs in the spinal cord of AL mice. S

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Figure 1.

Effects of 7,8-DHF treatment (5mg/kg, i.p.) on body weight and motor performance in ALS mice (SOD1^{G93A}). Starting at one month of age and until 105 days of age, body weight and motor performance were monitored twice a week. **A**, Body weight loss of ALS mice is not reversed by 7, 8-DHF. **B**, 7,8-DHF treatment improved motor performance of ALS mice up to the level of wild type (WT) mice (Repeated measures multi-way ANOVA, followed by the Dunns test for posthoc comparisons, * $p < 0.001$ as compared to the other groups, $n = 10$).

Figure 2.

Effect of 7,8-DHF treatment (5mg/kg, i.p.) on motor neurons count in the ventral horn of lumbar cord in ALS mice (SOD1^{G93A}). Stereological methods were employed to quantify the number of motor neurons. Neurons were marked with two different marker depending on size, 30μm diameter or larger and smaller than 30μm but larger than 15μm. **A, B, C, D**, Photomicrographs displaying Cresyl violet (CV) staining of WT-Saline (**A**), WT-7,8-DHF (**B**), ALS-Saline (**C**), and ALS-7,8-DHF (**D**). Magnifications for CV staining photomicrographs x10 (lower panels) and x60 (upper panels). Red arrows show MNs larger than 30μm, yellow arrows show MNs smaller than 30μm but larger than 15μm. **E, F, G**, MNs count in 7,8-DHF treated and untreated ALS mice (SOD1^{G93A}) and WT mice. **E**, 7,8-DHF treatment significantly increased total MNs count in ALS group. **F**, 7,8-DHF treatment significantly increased small MNs (15–30μm) count in ALS group. **G**, 7,8-DHF treatment did not change large MNs (>30μm) count in ALS group (One-way ANOVA, followed by Tukey's HSD test for multiple comparisons. * p<0.001 and #p<0.01, as compared to the other groups, n=10).

Figure 3.

Effect of 7,8-DHF treatment (5mg/kg, i.p.) on dendritic spine density of motor neurons in the ventral horn of lumbar cord in ALS mice (*SOD1^{G93A}*). Photomicrographs displaying Golgi staining of WT-Saline (A), WT-7,8-DHF (B), ALS-Saline (C), and ALS-7,8-DHF (D). Magnifications for photomicrographs x100. Spines were counted in 25 μm length of secondary branches branch for (red line in figure A). While untreated ALS mice (C) have lesser spines than WT, 7,8-DHF treated ALS mice (D) have as much as WT mice (A,B). E, 7,8-DHF treatment significantly increased spine density in ALS group (One-way ANOVA, followed by Tukey's HSD test for multiple comparisons. * $p < 0.001$, as compared to the other groups, $n = 10$).