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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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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 ALS mice.

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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.

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1. 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%

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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 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 [13–15] and BDNF potentiates neurotransmitter release in both developing neuromuscular synapses in culture [14–16], 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 ($SOD1^{G93A}$).

2. Materials and methods

2.1. 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.

2.2. 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) (5 mg/kg, i.p./3 days a week – dilution of DHF: 5 mg/ml – for each 20 g mouse approximately 0.02 ml) or saline (DHF's diluent).

2.3. 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.

2.4. Tissue preparation for post-mortem studies

Mice were euthanized at 105 days of age by CO_2 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 60 μm 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 NeuroTecnologies) following the manufacturer's instructions. Stained tissue was serially sectioned at 100 μm thickness using a cryostat microtome and used to quantify the spine density.

2.5. 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 ×4 magnification. The size of the counting frame was 75 μm × 75 μm and the area counted (XY) = 5625 μm^2. Cell counting was performed using a ×60 N.A 1.4 oil objective. Neurons were only counted if their diameter was 15 μm 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 μm long for easy detection of cells that meets the 15 μm diameter criterion. Neurons were marked with two different marker depending on size, 30 μm diameter or larger and smaller than 30 μm but larger than 15 μm. These MNs referred in text as small and large MNs, respectively.

2.6. Spine density

The density of dendritic spines was quantified by counting spines in 25 μm length of secondary branches of dendrite on selected motor neurons (Fig. 3A). Spine counting was performed