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24S-hydroxycholesterol suppresses neuromuscular transmission in SOD1(G93A) mice: A possible role of NO and lipid rafts

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by the initial denervation of skeletal muscle and subsequent death of motor neurons. A dying-back pattern of ALS suggests a crucial role for neuromuscular junction dysfunction. In the present study, microelectrode recording of postsynaptic currents and optical detection of synaptic vesicle traffic (FM1-43 dye) and intracellular NO levels (DAF-FM DA) were used to examine the effect of the major brain-derived cholesterol metabolite 24S-hydroxycholesterol (24S-HC, 0.4 μM) on neuromuscular transmission in the diaphragm of transgenic mice carrying a mutant superoxide dismutase 1 (SOD^{G93A}). We found that 24S-HC suppressed spontaneous neurotransmitter release and neurotransmitter exocytosis during high-frequency stimulation. The latter was accompanied by a decrease in both the rate of synaptic vesicle recycling and activity-dependent enhancement of NO production. Inhibition of NO synthase with L-NAME also attenuated synaptic vesicle exocytosis during high-frequency stimulation and completely abolished the effect of 24S-HC itself. Of note, 24S-HC enhanced the labeling of synaptic membranes with B-subunit of cholera toxin, suggesting an increase in lipid ordering. Lipid raft-disrupting agents (methyl-β-cyclodextrin, sphingomyelinase) prevented the action of 24S-HC on both lipid raft marker labeling and NO synthesis. Together, these experiments indicate that 24S-HC is able to suppress the exocytotic release of neurotransmitter in response to intense activity via a NO/lipid raft-dependent pathway in the neuromuscular junctions of SOD^{G93A} mice.

1. Introduction

A disturbance in synaptic communication lies at the heart of many neurodegenerative diseases and pharmacological approaches that act on the synaptic level are sought as a potential strategy for treatments. Amyotrophic Lateral Sclerosis (ALS) is a fatal disease leading to the progressive loss of both upper and lower motor neurons. Transgenic mice expressing a mutant copper/zinc superoxide dismutase 1 (SOD) gene develop a motor neuron disease resembling human ALS and they are commonly used as models for it (Gurney et al., 1994; Narai et al., 2009; Duplan et al., 2010; Gordon et al., 2010; Cappello et al., 2012; Rocha et al., 2013; Nascimento et al., 2015). Studies of SOD^{G93A} mice show a pattern of dying-back pathology (Nijssen et al., 2017). The earlier event in the ALS represents a denervation of neuromuscular junctions (NMJs) that occurs before the death of motor neurons (Fischer et al., 2004; Narai et al., 2009). Histological and electrophysiological studies reveal a similar pattern in ALS patients (Fischer et al., 2004; Nijssen et al., 2017). Together, these results suggest that NMJs are particularly vulnerable to damage in the progression of ALS, and suggest that this finally leads to diaphragm paralysis and respiratory failure, causing death. Early changes in neurotransmitter release were revealed at the NMJs in soleus muscle of SOD^{G37R} mice and in the diaphragms of SOD^{G93A} mice (Naumenko et al., 2011; Rocha et al., 2013; Nascimento et al., 2015; Arbour et al., 2015). In addition, a dysfunction of some proteins engaged in synaptic vesicle recycling and a reduction of the total size of the synaptic vesicle pool may be involved in ALS pathology (Cappello et al., 2012; Liu et al., 2015; Coyne et al., 2017).

ALS has also been found to be associated with marked changes in lipid metabolism, including cholesterol homeostasis (Wuolikainen et al., 2014; La Marca et al., 2016; Abdel-Khalik et al., 2017). According to some studies, hyperlipidemia is directly correlated with better survival in patients with ALS and lowering the levels of plasma cholesterol by statins may accelerate the disease progression (Dupuis et al., 2008;

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Zheng et al., 2013). Dyslipidemia and hypercholesterolemia may be accompanied by elevated plasma level of 24S-hydroxycholesterol (24S-HC), a major brain-derived cholesterol metabolite (Leoni and Caccia, 2013; Dumolt et al., 2017). In contrast to the CNS, where the concentration of 24S-HC varies in the range 10–30 μM (Lütjohann et al., 1996), the total plasma level of 24S-HC is about 0.2 μM, or 80 ng/ml in healthy volunteers (Babiker and Diczfalusy, 1998; Leoni and Caccia, 2013). The concentrations found in mouse plasma are lower than in human ranging from about 6 to 25 ng/ml in mice (Shafaati et al., 2010; Crick et al., 2015). Plasma level of 24S-HC may be significantly changed in some pathological conditions in a stage-dependent manner (Meng et al., 1997; Leoni and Caccia, 2013). In patients with some forms of neurodegeneration, an enhancement of 24S-HC production occurs in the early stage of the disease and then its synthesis is markedly reduced during the progressive neuronal loss (Leoni and Caccia, 2013; Petrov et al., 2017a). This may explain some discrepancies in measurements of 24S-HC. Wuolikainen et al. (2014) found that the plasma concentration of 24S-HC was increased (from 62 to 71 ng/ml) in females with ALS. By contrast, other studies revealed reduced levels of 24S-HC in cerebrospinal fluid and plasma (from 89 to 42 ng/ml) of ALS patients (La Marca et al., 2016; Abdel-Khalik et al., 2017).

Our recent study revealed that 24S-HC, at submicromolar range, could enhance synaptic vesicle cycling and affect retrograde NO signaling at the mouse NMJs (Kasimov et al., 2017). Given the dying-back pattern of pathology, early disturbance of NMJ functionality, respiratory dysfunction, and a potential implication of 24S-HC in the progression of ALS, it seems interesting to study the effect of 24S-HC on synaptic transmission in the diaphragm of SOD^{G93A} mice. Here, we report that 24S-HC (0.4 μM) suppresses both spontaneous release and evoked release during intense activity. The latter is dependent on both a decrease in the rate of synaptic vesicle retrieval and attenuation of activity-induced NO signaling via a lipid raft-dependent mechanism.

2. Methods

2.1. Ethical approval

This investigation conforms to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996) and European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No 123, Strasbourg, 1985). Experiments were performed on the isolated diaphragm muscle of SOD^{G93A} transgenic mice at the symptomatic disease stage (Gurney et al., 1994), characterized by paralysis of the hind limbs (6–7 months of age). Mice transgenic for the $SOD1^{G93A}$ mutation on a B6SJL background (B6SJL-Tg(SOD1-G93A)dl1Gur/J strain; purchased from The Jackson Laboratory) that harbor the mutated allele of the human SOD1 gene were maintained as a hemizygous line in an SPF-breeding facility (Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Pushino). The onset of the disease phenotype is delayed compared to the original high copy number strain (SOD1 G93A)1Gur, due to a decrease in transgenic copy number. Age-matched outbred B6/SJL mice, referred to as wild-type (WT), were used for comparison; they were exposed to the same conditions as the $SOD1^{G93A}$ mice. Mice had free access to food and water and were maintained in a 12-h light/12-h dark cycle.

Both male and female mice were used in equal proportions. Experiments without (control) and with application of 24S-HC were performed in tandem on both hemidiaphragms. Animals were anesthetized by an intraperitoneal injection of sodium pentobarbital (40 mg/kg) before decapitation with a guillotine. The chest was then immediately opened and the muscle quickly excised. The experimental protocol met the requirements of the EU Directive 2010/63/EU and was approved by the Bioethics Committees of Kazan Medical University.

2.2. Bathing solution and chemicals

Hemidiaphragms with a phrenic nerve were attached to the bottom of a Sylgard-lined chamber (volume, 5 ml), which was superfused at 5 $ml \cdot min^{-1}$ throughout the experiment with mammalian physiological saline containing (in mM): NaCl-129.0, KCl-5.0, $CaCl_2$-2.0, $MgSO_4$-1.0, NaH_2PO_4-1.0, $NaHCO_3$-20.0, glucose-11.0 and HEPES-3.0. The solution was saturated with a 5% CO_2 and 95% O_2 mixture to maintain pH. Experiments were performed at 24–25 °C which allows the muscle to maintain a stable level of neurotransmitter release for a long period (Glavinović, 1979) and to prevent rapid leakage of fluorescent dye (DAF-FM) from the cytosol (Pye et al., 2007). The phrenic nerve was stimulated with a suction electrode connected to an extracellular stimulator (DS3 Digitimer Ltd., UK) by supramaximal electrical pulses of 0.1 ms duration. In some experiments (electrophysiology and FM1-43/DAF-FM fluorescence), the muscle fibers were cut transversely to prevent muscle contractions and to simultaneously maintain the physiological level of quantal release of acetylcholine.

24S-HC (Enzo Life Sciences) was dissolved in DMSO (the final concentration of DMSO in the working solution did not exceed 0.001%). The concentration of 24S-HC was 0.4 μM, which is sufficient to evoke a submaximal effect on synaptic vesicle exocytosis at the NMJs of WT mice (Kasimov et al., 2017). Preparations were treated with 0.4 μM 24S-HC for 15 min before high-frequency stimulation. DMSO (0.001%) by itself did not alter any of the measured parameters (spontaneous or evoked postsynaptic currents; labeling with FM1-43, DAF-FM and CTxB), consistent with our previous observation (Kasimov et al., 2017). Therefore the data from DMSO controls were pooled together with DMSO-free controls.

Nω-nitro-L-arginine methyl ester hydrochloride (100 μM L-NAME, an inhibitor of NO synthase) was added to the external solution 30 min before 24S-HC treatment and remained in the bath throughout the experiment. In some experiments, we used a 10 min treatment with methyl-β-cyclodextrin (MβCD, 0.1 mM) or sphingomyelinase (SMase, 0.1 μ/ml; from *Staphylococcus aureus*, Sigma) to disrupt lipid rafts. Under these conditions, MβCD or SMase was added after 15 min application of 24S-HC. The osmolarity of the solution was adjusted when the reagents were added to physiological solution.

2.3. Electrophysiology

Recording of postsynaptic end-plate currents (EPCs) and miniature EPC (MEPCs) was performed using standard two-electrode voltage clamp technique with intracellular glass microelectrodes (tip diameter ~1 μm, resistance 3-5MΩ, filled with 2.5 M KCl). The junctional zone was located between two microelectrodes separated by distance of ~200–300 μm. The holding potential for the cut muscle fibers was kept at −45 mV (leak current in the range of 10–30 nA, ≤10% of EPC amplitudes). The motor nerve was stimulated by single (1 stimulus in 20 s) or high-frequency trains (20 Hz) of suprathreshold pulses. The recorded signals were digitized at 50 kHz and analyzed off-line using PC software. Recording instrumentation consisted of an Axoclamp 900 A (Molecular devices, USA) amplifier and LA II digital I/O board (Pushino, Russia) under the control of locally written software.

2.4. Fluorescence microscopy

Fluorescence images were acquired using an Olympus BX51WI microscope with a confocal attachment Disk Speed Unit and LumPlanPF 100 × w objective. Images were captured with DP71 CCD camera (Olympus). Image analysis was performed using CellˆP (Olympus) and ImagePro software (Media Cybernetics, Bethesda, MD, USA). Multiple z axis optical sections were taken using a focus stepper (ECO-MOT). Intensity analysis was made on regions of interest in arbitrary units (a.u.), which were then converted into percentages. Only nerve terminals on surface muscle fibers were studied. All dyes were from