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Regular Article

Fluoxetine is neuroprotective in slow-channel congenital myasthenic syndrome

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ABSTRACT

The slow-channel congenital myasthenic syndrome (SCS) is an inherited neurodegenerative disease that caused mutations in the acetylcholine receptor (AChR) affecting neuromuscular transmission. Leaky AChRs lead to Ca^{2+} overload and degeneration of the neuromuscular junction (NMJ) attributed to activation of cysteine proteases and apoptotic changes of synaptic nuclei. Here we use transgenic mouse models expressing two different mutations found in SCS to demonstrate that inhibition of prolonged opening of mutant AChRs using fluoxetine not only improves motor performance and neuromuscular transmission but also prevents Ca^{2+} overload, the activation of cysteine proteases, calpain, caspase-3 and 9 at endplates, and as a consequence, reduces subsynaptic DNA damage at endplates, suggesting a long term benefit to therapy. These studies suggest that prolonged treatment of SCS patients with open ion channel blockers that preferentially block mutant AChRs is neuroprotective.

Introduction

The slow-channel congenital myasthenic syndrome (SCS) is a degenerative neuromuscular disorder characterized by generalized fatigability, weakness, and wasting of face and limb muscles caused by point mutations in the muscle acetylcholine receptor (AChR). Mutant AChRs lead to disturbed gating, prolonged channel open times, postsynaptic Ca^{2+} overload, and degeneration of the neuromuscular junction (NMJ) (Engel et al., 1982; Gomez et al., 1996, 2002a, 2002b). Previously, we showed that mutant AChRs are leaky and lead to Ca^{2+} overload of the postsynaptic region in conjunction with Ca^{2+} release from internal stores through the sarcoplasmic reticulum-resident type 1 inositol 1,4,5-triphosphate receptor (IP_3R_1) channel (Zayas et al., 2007; Zhu et al., 2011). Localized Ca^{2+} overload gives rise to both functional and structural impairment of the NMJ and neuromuscular transmission (Zhu et al., 2011, 2013, 2014; Groshong et al., 2007). Therefore, prevention of prolonged opening of a mutant AChR channel is a logical strategy in treatment of SCS.

Recently, both quinidine, and the anti-depressant, fluoxetine, have been shown to be of benefit in SCS by preferentially blocking mutant AChRs, apparently normalizing prolonged synaptic currents (Fukudome et al., 1998; Harper et al., 2003). Fluoxetine reduces the prolonged opening of mutant AChR channels in vitro by several fold compared to wild-type and improves neuromuscular transmission in SCS (Harper et al., 2003; Chaouch et al., 2012; Colomer et al., 2006). These reports demonstrate that inhibition of mutant AChR channel with fluoxetine functionally improves muscle strength and performance in SCS patients.

Detailed quantitative and structural outcome studies are difficult in humans. In this study, we used a well-established transgenic mouse model of SCS (mSCS) to assess the long-term benefits of fluoxetine therapy to functional and structural aspects of the NMJ. We showed that fluoxetine treatment remarkably attenuates the characteristic decrements of compound muscle action potentials associated with SCS and functionally improves quantitative measures of gait in mSCS. More importantly fluoxetine reduces Ca^{2+} overload at

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endplates, decreases activation of caspase-3 and -9 at the NMJ, and diminishes DNA damage in subsynaptic nuclei. These studies provide experimental evidence for the long-term, neuroprotective benefit of fluoxetine in SCS.

Material and methods

Materials

Chemical reagents were purchased from Sigma Chemical Co. Cell culture materials were obtained from Gibco BRL Co. Laminin, Texas Red conjugated alpha-bungarotoxin (αBT) (TxR-αBT), and fluorescence-tagged secondary antibodies were purchased from Invitrogen Co. Secondary antibodies with horseradish peroxidase (HRP) were provided by GE Healthcare Bioscience Co. Primary antibodies were used to the following targets: phosphorylated H2AX (Ser139; 1:500; Millipore) and cleaved caspase-3 and -9 (1:200; Cell Signaling).

Animals and tissue preparation

4–6 month-old male wild type FVB mice and SCS transgenic mice (mSCS; εL269F and δS268F) were used in this study (Gomez et al., 1997). Both were derived by targeted expression of the respective mutant cDNA to muscle using the muscle creatine kinase promoter (Gomez et al., 1997). Both εL269F and δS268F mutations have been reported in patients with SCS (Gomez et al., 1998, 2002b). The clinical electrophysiological and pathological features of the transgenic mouse lines have been described in detail elsewhere (Gomez et al., 1997, 2002a; Zayas et al., 2007; Vohra et al., 2006). All drug treatments and surgical procedures followed the animal care and use protocols established by Institutional Animal Care and Use Committee (IACUC). Mice were anesthetized using ketamine and xylazine (Mulder and Mulder, 1979).

Drug administration

Fluoxetine hydrochloride (Sigma) in saline (0.9% w/v; Fisher Thermoscientific, Lake Forest, US) was administered intraperitoneally at the series of concentration of 2.4, 4.8, 7.2 and 9.6 μg/g daily to different mSCS groups for 9 days, respectively. Excised diaphragm of mSCS mice was treated in vitro with fluoxetine as 0.3, 0.9 and 1.5 μM.

Tissue staining and imaging

Motor endplates in the serial sectioned TA muscle (tibialis anterior) were localized using a histochemical stain for cholinesterase (Koelle and Friedenwald, 1949) and Ca^{2+}-overloaded endplates were detected by glyoxal bis 2 hydroxyanil (GBHA) stain as described in (Kashiwa and House, 1964; Koelle and Friedenwald, 1949). For immunohistochemistry, tissues sections were fixed in a 1:1 methanol-acetone mixture at −20 °C for 30 min, air dried for 20 min, and incubated with blocking solution for 1 h at RT, followed by overnight incubation with primary antibody at 4 °C. After washing, fluorescent secondary antibody in PBS-T (phosphate buffered saline and 0.05% Tween-20) for 1 h at RT. Confocal fluorescence microscopy was carried out under a TCS laser scanning microscope (Leica, Deerfield, IL). Optical sections of 0.5 μm were scanned for the *z*-axis. Image J was used to quantify the percentage of specific expression of antibody in NMJs of each sample.

Quantitative studies in tissue sections

Quantitation of the proportion of labeled NMJs in the TA muscle cryosections was carried out using sequential alternate sections stained for cholinesterase and test label (GBHA, pH2X and caspase 3 and 9) as described previously (Gomez et al., 1997, 2002a), using Image J (v5.3) for analysis.

Caspase activity assay

Caspase-3 and caspase-9 activities in muscle were measured using a firefly luciferase-based assay (Calpain-Glo™ Protease Assay; Caspase-Glo® 3/7 and 9 Assays; Promega). Muscle samples were homogenized as previously described (Lee et al., 2008) except for the addition of 10 mM NH_4Cl and 10 mM 3-methyladenine to the homogenization buffer to stabilize lysosomes and the proteasome complexes. Protein samples (20 μg) were analyzed by luminometer (Turner BioSystems, Inc) in triplicate.

Electromyography (EMG)

Compound muscle action potentials (CMAPs) were detected and calculated as described previously using a Nicolet VikingQuest (Nicolet Biomedical, Inc) (Groshong et al., 2007).

Electrophysiology

Two-electrode voltage clamp (TEVC) recordings of excised mouse diaphragm were performed as described (Gomez et al., 1997, 2002b; Zhu et al., 2011). Briefly, after dissection diaphragm muscle was continuously perfused with a Tyrode's solution with a composition (in mM): 137 NaCl, 2.8 KCl, 1.8 $CaCl_2$, 1.1 $MgCl_2$, 11.9 $NaHCO_3$, 0.33 NaH_2PO_4, and 11.2 dextrose, pH 7.4 and pinned to a SYLGARD (Dow Corning Corporation, Midland, MI) chamber containing the Tyrode's solution and bubbled with a mixture of 95% O_2 and 5% CO_2.

Intracellular potentials and currents were recorded using an Axon900A (Molecular Devices, Sunnyvale, CA) amplifier and borosilicate microelectrodes were prepared using the Flaming/Brown Micropipette Puller Model P-87 (Sutter Instruments), filled with a solution 3 M KCl and were later beveled using the BV-10 Micro-Pipette Beveler (Sutter Instruments) to improve morphology and clamp efficiency with a resistance raging from 5 to15 MΩ choosing the lowest in resistance for the current electrode. Using the TEVC, miniature endplate currents (MEPCs) were recorded at a holding potential of −70 mV. The output of the recording instrument was filtered at 1 kHz analog to digital converter using a Digidata 1440A (Molecular Devices, Sunnyvale, CA). Currents were digitized at 100 μs per point and stored, captured and analyzed using pClamp10.3 software (Molecular Devices, Sunnyvale, CA). The miniature end plate currents were analyzed using the mini analysis program (Synaptosoft Inc., Fort Lee New Jersey). Finally, the amplitude, time constant and frequency for the recorded MEPCs were fitted and graphed to address the open channel blocker effect of fluoxetine on SCS panel. Endplates from untreated εL269F SCS transgenic mice with WT-like miniature kinetics were excluded from further analysis.

Treadmill gait analysis

Mouse gait analysis was performed via the DigiGait Imaging System (Mouse Specifics, Inc., Boston) as described previously (Zhu et al., 2011). Briefly, mice walked on a motor-driven treadmill with a transparent treadmill belt. A high frame rate camera was focused on the ventral plane of the mice as they walked within an acrylic chamber, ~5 cm wide by ~25 cm long. Due to the remarkable reduction of muscle strength in mSCS, brake time was primarily selected as the most sensitive metric to demonstrate functional improvement of treated muscle in mSCS. The treadmill speed was set to 25 cm/s. Approximately 5 s of video was collected for each walking mouse to provide ~20 sequential strides. The right hind limb from every mSCS was measured at pre- and post-treatment with fluoxetine, in which mSCS administrated with saline was selected as control group.