



## 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<sup>2+</sup> 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<sup>2+</sup> 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.

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## 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<sup>2+</sup> 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<sup>2+</sup> overload of the postsynaptic region in conjunction with Ca<sup>2+</sup> release from internal stores through the sarcoplasmic reticulum-resident type 1 inositol 1,4,5-triphosphate receptor (IP<sub>3</sub>R<sub>1</sub>) channel (Zayas et al., 2007; Zhu et al., 2011). Localized Ca<sup>2+</sup> 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<sup>2+</sup> 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 ( $\alpha$ BT) (TxR- $\alpha$ BT), 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;  $\epsilon$ L269F and  $\delta$ S268F) 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  $\epsilon$ L269F and  $\delta$ S268F 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  $\mu$ g/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  $\mu$ M.

### 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  $\text{Ca}^{2+}$ -overloaded endplates were detected by glyoxal bis 2 hydroxylanil (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  $-20^\circ\text{C}$  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  $4^\circ\text{C}$ . 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  $\mu$ m 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  $\text{NH}_4\text{Cl}$  and 10 mM 3-methyladenine to the homogenization buffer to stabilize lysosomes and the proteasome complexes. Protein samples (20  $\mu$ g) 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).

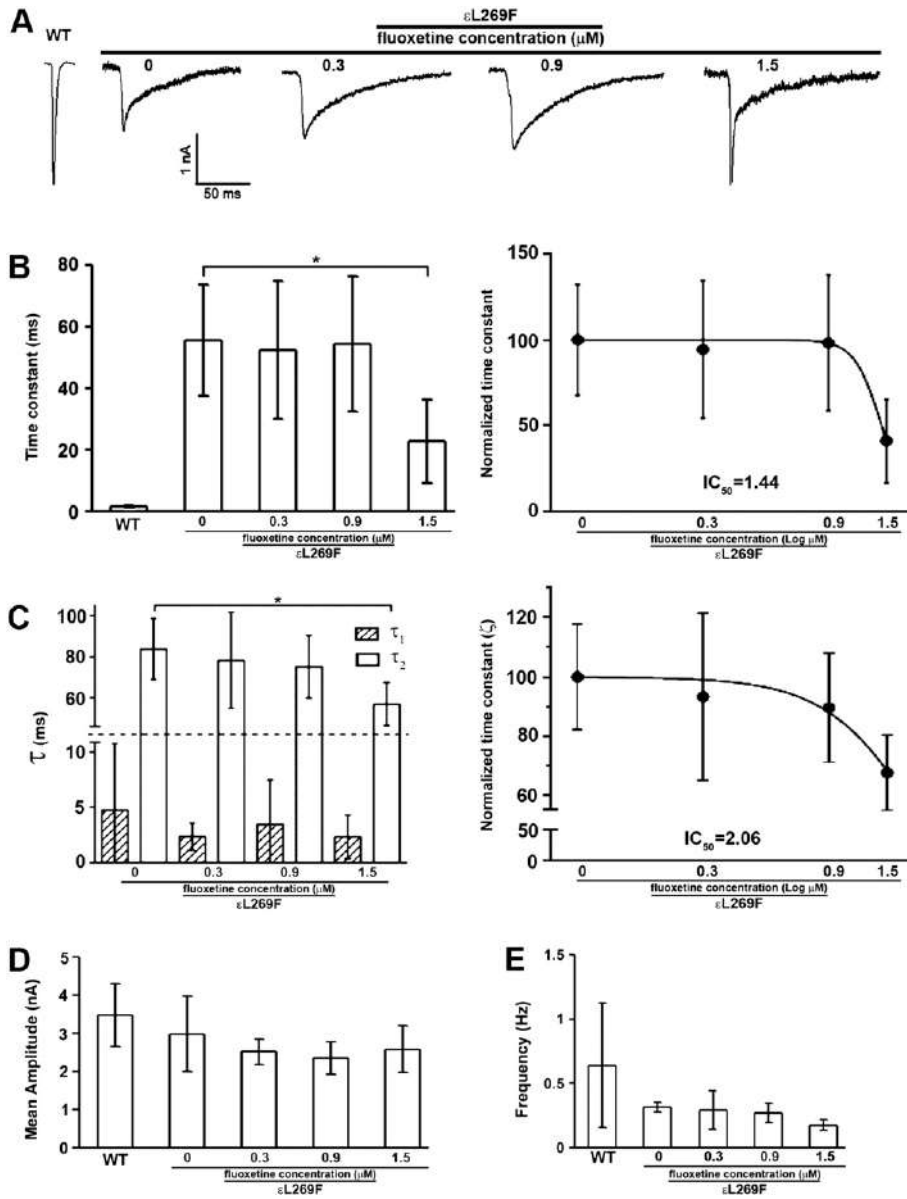
### Electrophysiology

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  $\text{CaCl}_2$ , 1.1  $\text{MgCl}_2$ , 11.9  $\text{NaHCO}_3$ , 0.33  $\text{NaH}_2\text{PO}_4$ , 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%  $\text{O}_2$  and 5%  $\text{CO}_2$ .

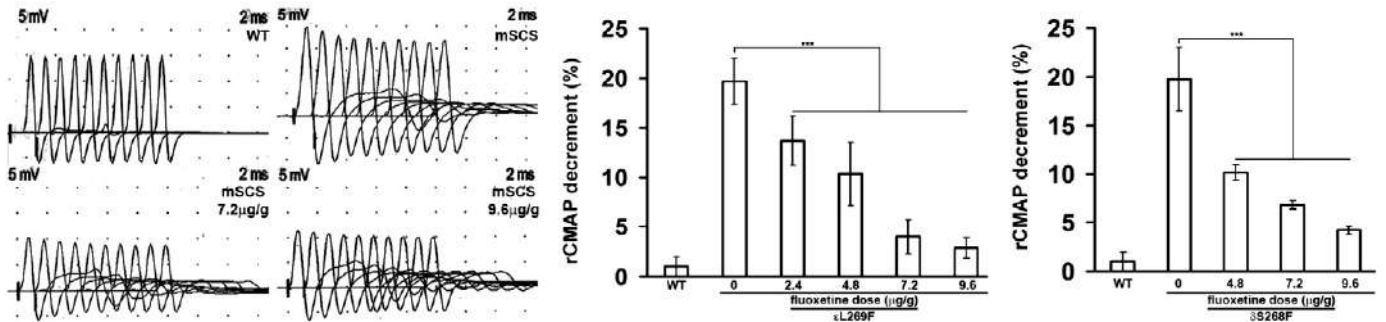
Intracellular potentials and currents were recorded using an Axon900A (Molecular Devices, Sunnyvale, CA) amplifier and borosilicate microelectrodes were prepared using the Flaming/Brown Micropipette Puller Model P-87 (Sutter Instruments), filled with a solution 3 M KCl and were later beveled using the BV-10 Micro-Pipette Beveler (Sutter Instruments) to improve morphology and clamp efficiency with a resistance ranging from 5 to 15 M $\Omega$  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  $\mu$ s 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  $\epsilon$ L269F 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,  $\sim 5$  cm wide by  $\sim 25$  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  $\sim 20$  sequential strides. The right hind limb from every mSCS was measured at pre- and post-treatment with fluoxetine, in which mSCS administered with saline was selected as control group.



**Fig. 1.** Fluoxetine treatment corrects altered mSCS  $\epsilon$ L269F nAChR kinetics. Two-electrode voltage clamp (TEVC) recordings of quantal activity for wild type and  $\epsilon$ L269F mice diaphragms treated acutely with fluoxetine at concentrations of 0.3, 0.9 and 1.5  $\mu$ M. A. The decay time constant in control SCS transgenic mice was  $55.60 \pm 17.95$  ms whereas  $52.48 \pm 22.26$  in 0.3  $\mu$ M treatment,  $54.56 \pm 22.04$  in 0.9  $\mu$ M treatment and  $22.80 \pm 13.59$  in 1.5  $\mu$ M treatment. B. The MEPC time constant was dramatically reduced in SCS transgenic mice with 1.5  $\mu$ M treatment. n = 4, \* $p < 0.05$ . Student's  $t$ -test. C. Decay phases were resolved into two exponents ( $\tau_1$  and  $\tau_2$ ) with  $\tau_2$  showing a concentration dependent reduction. n = 4, \* $p < 0.05$ . Student's  $t$ -test. D and E. Neither mean amplitude nor the frequency of MEPCs recorded from  $\epsilon$ L269F mice was affected by fluoxetine. n = 4; Student's  $t$ -test.



**Fig. 2.** Decremental responses in rsEMG in mSCS with fluoxetine treatment. Left. Compound muscle action potentials (CMAP) recorded during repetitive stimulation (rsEMGs) in WT, untreated mSCS and mSCS treated with fluoxetine. Decremental responses were seen in mSCS while reduced decremental responses were shown in mSCS ( $\epsilon$ L269F) with fluoxetine treatment of 7.2 and 9.6  $\mu$ g/g. Middle.  $\epsilon$ L269F mice with 2.4  $\mu$ g/g fluoxetine reduced decrement to  $13.6 \pm 2.5\%$  whereas decrement was  $10.3 \pm 3.2\%$  with 4.8  $\mu$ g/g fluoxetine,  $4.0 \pm 1.7\%$  with 7.2  $\mu$ g/g fluoxetine, and  $2.8 \pm 1.0\%$  with 9.6  $\mu$ g/g treatment, compared with  $19.7 \pm 2.3\%$  in untreated  $\epsilon$ L269F mice. Right. To treated  $\delta$ S268F, decrement was  $10.9 \pm 1.2\%$  after 4.8  $\mu$ g/g fluoxetine,  $6.8 \pm 0.5\%$  after 7.2  $\mu$ g/g, and  $4.2 \pm 0.6\%$  after 9.6  $\mu$ g/g treatment, compared with  $18.8 \pm 3.1\%$  in untreated  $\delta$ S268F mice. n = 7; \*\*\* $p < 0.001$ , all comparisons were analyzed by Student's  $t$ -test.

## Statistics

Values were represented as the mean  $\pm$  STDEV. Data were analyzed using Student's *t*-test and Chi-square test where appropriate, and non-parametric Mann–Whitney *u*-test, for comparisons of percentages and ratio values, as indicated in figure legends.

## Results

### Fluoxetine lowers the $\epsilon$ L269F MEPC time constant in vitro

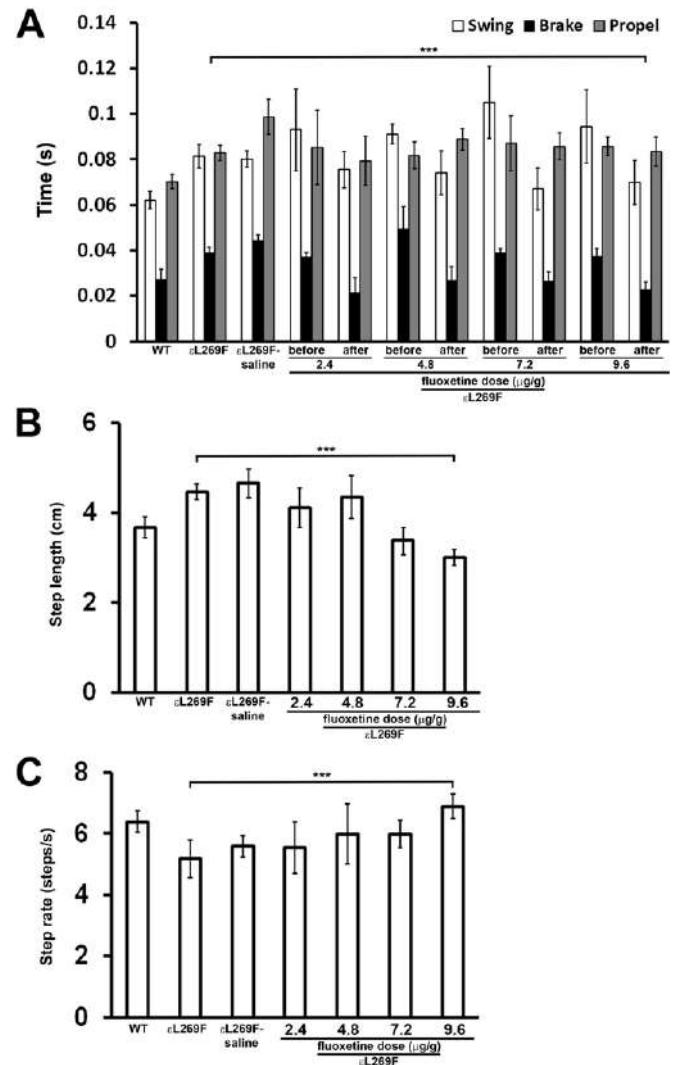
We investigated the effect of fluoxetine on neuromuscular transmission in mSCS mice using TEVC by acutely exposing voltage-clamped muscle fibers to a range of physiological fluoxetine concentrations (Amsterdam et al., 1997; Goodnick, 1991; Grajales-Reyes et al., 2013), 0.3, 0.9 and 1.5  $\mu$ M by bath application. As reported previously, decay phases of MEPCs from untreated mSCS mice were nearly eight-fold longer than control mice and MEPC amplitudes were reduced by 31%. After application of fluoxetine MEPCs showed a concentration-dependent reduction in the time constant ( $\tau_s$ ), which averaged to  $55.60 \pm 17.95$ ,  $52.48 \pm 22.26$ ,  $54.56 \pm 22.04$  and  $22.80 \pm 13.59$  ms, in mock-treated, 0.3, 0.9 and 1.5  $\mu$ M respectively (Figs. 1 A, B). The decay phases could be resolved into two exponents ( $\tau_1$  and  $\tau_2$ ) with  $\tau_2$  showing a concentration dependent reduction, which averaged in a control SCS transgenic mice  $83.82 \pm 14.99$  ms, whereas  $78.20 \pm 23.57$  in 0.3  $\mu$ M treatment,  $75.17 \pm 15.47$  in 0.9  $\mu$ M treatment and  $56.75 \pm 10.63$  in 1.5  $\mu$ M treatment (Fig. 1 C). Fluoxetine concentrations that reduced MEPC decay time by 3.3-fold did not affect the MEPC amplitude (Fig. 1D). Similarly, the MEPC frequencies remained unaffected by the treatment since no significant difference could be detected between the untreated and fluoxetine-treated at three concentrations (Fig. 1E). These results suggest that fluoxetine treatment in vitro significantly reduces open time of mutant nAChR channels without influencing other physiological properties.

### Fluoxetine improves neuromuscular transmission and functional recovery in SCS transgenic mice

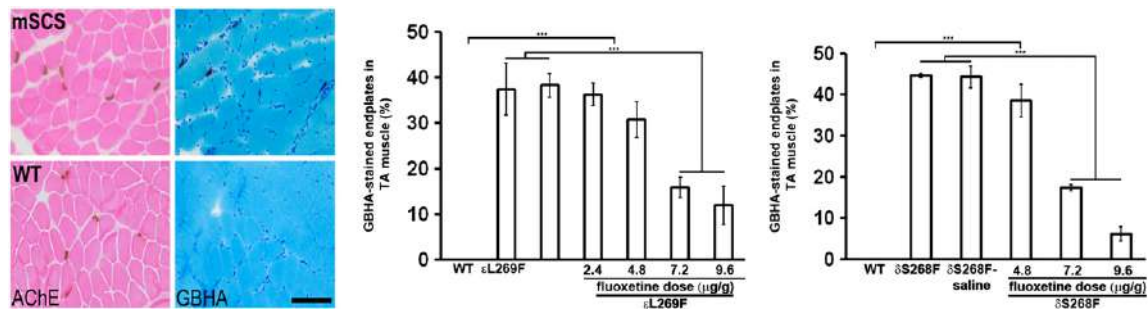
Weakness and fatigability in SCS and other NMJ disorders arise from impaired synaptic transmission associated with decremental compound muscle action potential (CMAP) amplitudes evoked by repetitive nerve stimulation (rsEMG). mSCS mice expressing different SCS mutations exhibit decremental responses to rsEMG (Gomez et al., 1997, 2002a, 2002b; Zhu et al., 2011). We investigated whether fluoxetine treatment would improve decrement in rsEMG. 9 days after fluoxetine treatment, we found a dose-dependent reduction in decrement of rsEMG in mSCS mice. Treatment of  $\epsilon$ L269F mice with 2.4  $\mu$ g/g fluoxetine led to a mean decrement of  $13.6 \pm 2.5\%$ ; with a dose of 4.8  $\mu$ g/g the decrement was  $10.3 \pm 3.2\%$ ; with 7.2  $\mu$ g/g, the decrement was  $4.0 \pm 1.7\%$ ; and with 9.6  $\mu$ g/g mean decrement was  $2.8 \pm 1.0\%$ , compared with  $19.7 \pm 2.3\%$  in untreated  $\epsilon$ L269F mice. Similar results were also seen in treated  $\delta$ S268F, in which reduction of decrement was  $10.9 \pm 1.2\%$  after 4.8  $\mu$ g/g,  $6.8 \pm 0.5\%$  after 7.2  $\mu$ g/g, and  $4.2 \pm 0.6\%$  after 9.6  $\mu$ g/g treatment, compared with  $18.8 \pm 3.1\%$  in untreated  $\delta$ S268F mice (Fig. 2;  $n = 7$ ;  $p < 0.001$ ).

To investigate whether fluoxetine treatment led to improved motor performance in mSCS muscle, we used a computerized, video-assisted treadmill (DigiGait<sup>®</sup>, MouseSpecifics), which allows comparison of motor behavior using individual limb of each mouse with several performance parameters, including: “brake time”, “stride length”, and “stride frequency”. Previous studies showed that “brake time” was the most sensitive parameter for detection of improved muscle function in mSCS  $\epsilon$ L269F with different treatments (Zhu et al., 2011). We tested whether fluoxetine treatment of mSCS  $\epsilon$ L269F mice, the more severely affected mouse line, improved motor function, with evaluations standardized to the right hind limb. We found that fluoxetine treatment

significantly reduced brake time, to nearly half of pre-treatment measurements ( $23 \pm 4$  vs  $37 \pm 3$  ms;  $p < 0.001$ ) to a level comparable to that of WT mice (Fig. 3A;  $n = 7$ ). The stride length in untreated mSCS mice was 4.5 cm compared with 3.7 cm in WT mice ( $n = 6$ ;  $p < 0.001$ ). Fluoxetine treatment significantly reduced the mean stride length to 3.0 cm at the highest dose (Fig. 3B;  $n = 7$ ;  $p < 0.001$ ). The mean stride frequency in mSCS mice was  $5.2 \text{ s}^{-1}$  compared with  $6.4 \text{ s}^{-1}$  in WT mice ( $n = 7$ ;  $p < 0.001$ ). Fluoxetine increased the mean stride frequency to  $6.9 \text{ s}^{-1}$  in mSCS (Fig. 3C;  $n = 7$ ;  $p < 0.001$ ). These findings demonstrate the functional benefit of fluoxetine treatment to the neuromuscular system by improved neuromuscular transmission



**Fig. 3.** Fluoxetine treatment improves motor function in mSCS ( $\epsilon$ L269F). A. Right hindlimb motor time interval parameters measured in motorized treadmill (DigiGait<sup>®</sup>) in WT and mSCS and mSCS treated fluoxetine. Functional parameters termed “swing”, “brake” and “propel” are time intervals for three components of normal leg movements recorded doing walking, as defined in methods are identical between left and right hindlimbs in WT mice. All three parameters are symmetrically and significantly increased at right hindlimbs in pre-treated mSCS mice and control mSCS treated with saline (mSCS-saline) in comparison to WT. After fluoxetine treatment, brake time from the identical pre-treated hindlimbs is significantly reduced in treated mSCS whereas no statistical difference is found in the time of propel and swing.  $n = 7$ ;  $***p < 0.001$ , Student's *t*-test. B. Mean hindlimb stride length in WT and mSCS and mSCS treated with fluoxetine. The average stride length for both limbs in mSCS ( $4.46 \pm 0.18$ ) and mSCS control ( $4.65 \pm 0.31$ ) is significantly longer than WT ( $3.67 \pm 0.24$ ). Fluoxetine treatment reduces mean stride length to  $3.0 \pm 0.17$  in treated mSCS.  $n = 7$ ;  $***p < 0.001$ , Student's *t*-test. C. Mean step frequency in WT and mSCS and mSCS treated with fluoxetine. The average step frequency for both limbs in mSCS ( $5.18 \pm 0.62$ ) and mSCS control ( $5.58 \pm 0.35$ ) is significantly lower than WT ( $6.38 \pm 0.34$ ). Fluoxetine treatment increases mean step frequency to  $6.88 \pm 0.4$  in treated mSCS.  $n = 7$ ;  $***p < 0.001$ , Student's *t*-test.



**Fig. 4.** Fluoxetine prevents  $\text{Ca}^{2+}$  overload of NMJs in mSCS. Left. Serial TA muscle sections labeled for cholinesterase (dark brown) to localize endplates and GBHA (red) to localize  $\text{Ca}^{2+}$  overloading in WT and mSCS.  $\text{Ca}^{2+}$  overloading was found at endplates of mSCS, but not in wild type (WT). Scale bar = 60  $\mu\text{m}$ . Middle. Quantitation of  $\text{Ca}^{2+}$ -overload of endplates (% GBHA-labeled endplates) in WT and in untreated mSCS mice ( $\epsilon\text{L269F}$ ) and with treatment of serial concentrations of fluoxetine. Endplates from WT TA showed no  $\text{Ca}^{2+}$ -labeled endplates, while a significant number of NMJ were overloaded with  $\text{Ca}^{2+}$  in untreated  $\epsilon\text{L269F}$  ( $37.3 \pm 5.7\%$ ) and control  $\epsilon\text{L269F}$  treated with saline (mSCS ( $\epsilon\text{L269F}$ )-saline;  $38.2 \pm 2.6\%$ ) whereas  $\text{Ca}^{2+}$ -overloaded endplates were significantly decreased in 7.2  $\mu\text{g/g}$  ( $15.8 \pm 2.3\%$ ) and 9.6  $\mu\text{g/g}$  ( $11.9 \pm 4.2\%$ ) treatment. Right. The similar results were also shown in mSCS mice ( $\delta\text{S268F}$ ). Compared with  $\delta\text{S268F}$  ( $44.7 \pm 0.5\%$ ) and control  $\delta\text{S268F}$  ( $44.3 \pm 2.7\%$ ), treated  $\delta\text{S268F}$  with a dose of 7.2 and 9.6  $\mu\text{g/g}$  reduced  $\text{Ca}^{2+}$ -labeled endplates to  $17.4 \pm 0.8\%$  and  $6.1 \pm 1.7\%$ , respectively.  $n = 5-7$ ;  $***p < 0.001$ . All comparisons were analyzed by Student's *t*-test.

in SCS, although tissue studies are needed to explore the neuroprotective effect of fluoxetine.

#### Fluoxetine inhibits pathological $\text{Ca}^{2+}$ overload at endplates in mSCS

Based on the effect of fluoxetine on mutant AChR gating we hypothesized that this drug would reduce pathological accumulation of postsynaptic  $\text{Ca}^{2+}$  at the NMJ (Gomez et al., 2002a; Zhu et al., 2011). We administered fluoxetine intraperitoneally daily to mSCS mice ( $\epsilon\text{L269F}$  and  $\delta\text{S268F}$ ) over a range of doses (2.4, 4.8, 7.2, and 9.6  $\mu\text{g/g}$ ). Control mSCS mice were treated with saline or not at all. Nine days later, we examined  $\text{Ca}^{2+}$  overload in TA muscle sections using the glyoxal bis 2-hydroxyanil (GBHA) histochemical stain (Kawabuchi, 1982; Leonard and Salpeter, 1979).  $\text{Ca}^{2+}$  was present at mSCS endplates, but not in WT TA muscle (Fig. 4; left). After a 9-day treatment of  $\epsilon\text{L269F}$ ,  $\text{Ca}^{2+}$ -overloaded endplates were significantly decreased in 7.2  $\mu\text{g/g}$  ( $15.8 \pm 2.3\%$ ) and 9.6  $\mu\text{g/g}$  ( $11.9 \pm 4.2\%$ ), compared with  $38.2 \pm 2.6\%$  in control group (Fig. 4; middle;  $n = 5$ ;  $p < 0.001$ ). Similar results were also observed in  $\delta\text{S268F}$  mice, in which GBHA-labeled endplates were reduced to  $17.4 \pm 0.8\%$  and  $6.1 \pm 1.7\%$  in 7.2  $\mu\text{g/g}$  and 9.6  $\mu\text{g/g}$  treatments, respectively, compared with  $44.3 \pm 2.7\%$  in control group (Fig. 4; right;  $n = 7$ ;  $p < 0.001$ ). Other doses have no effects on mSCS muscle. These results suggest that reducing open time of mutant AChRs significantly reduces  $\text{Ca}^{2+}$  overload at NMJs in mSCS muscle.

#### Fluoxetine prevents cysteine protease activation in mSCS

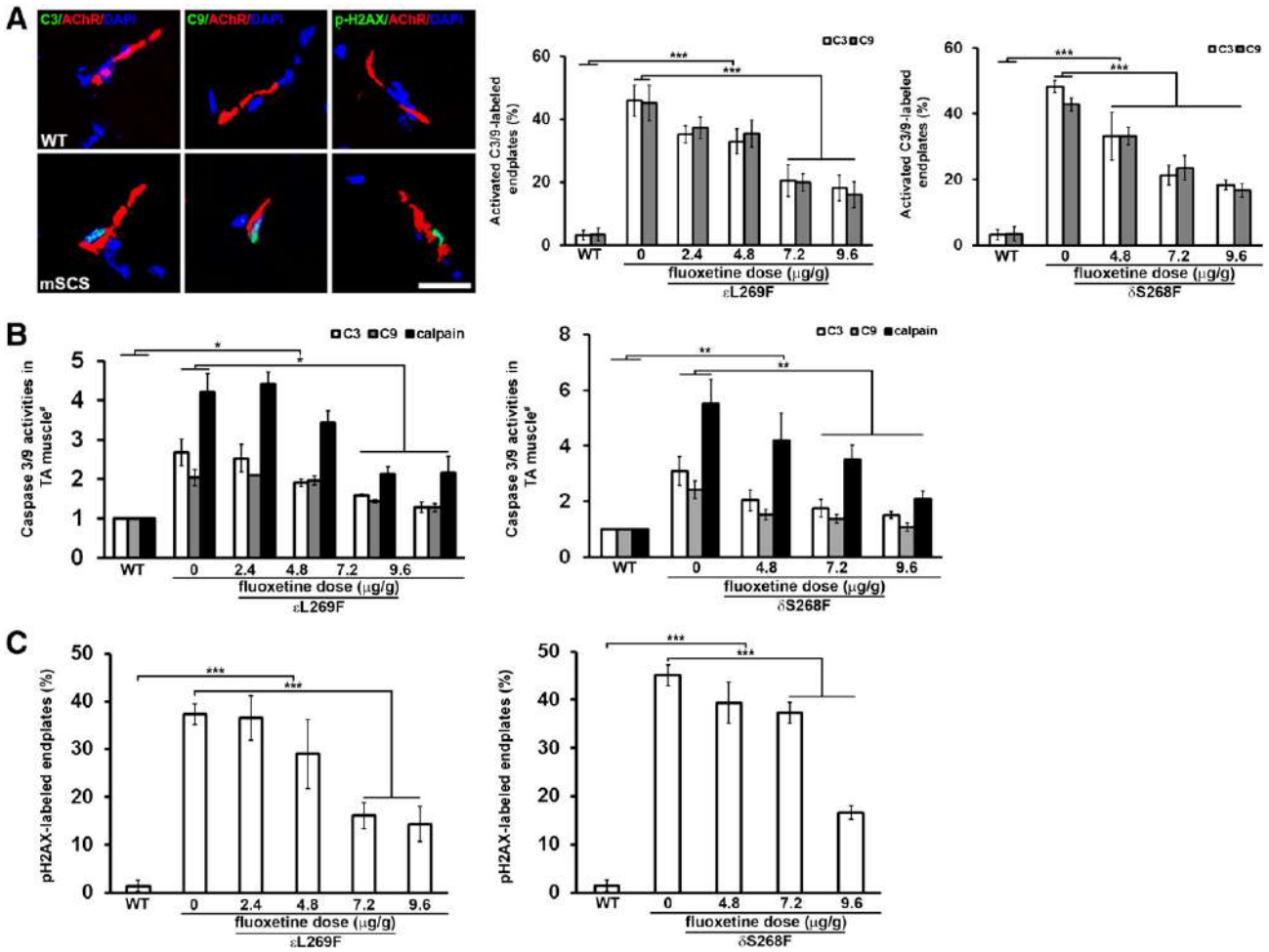
We reported previously that both calpain and caspase proteases are activated at NMJs in mSCS, to an extent comparable to the endplate  $\text{Ca}^{2+}$  overload (Zhu et al., 2011, 2013, 2014; Groshong et al., 2007). Therefore, we hypothesized that fluoxetine would also reduce these protease activities in mSCS muscle. We found that fluoxetine significantly reduced the accumulation of active caspases in mSCS muscle. In untreated  $\epsilon\text{L269F}$  mice, 45.9% and 45.2% of endplates were labeled intensely for active caspase-3 and active caspase-9, respectively. In  $\epsilon\text{L269F}$  mice treated with increasing doses of fluoxetine, activated caspases were detected at progressively fewer endplates, from 35.2% and 37.3% for caspase-3 and caspase-9, respectively, and at a dose of 2.4  $\mu\text{g/g}$  to 18.2% and 16.1% caspase-3 and caspase-9, respectively at a dose of 9.6  $\mu\text{g/g}$  (Fig. 5A;  $p < 0.001$ ). Similar results were also found in  $\delta\text{S268F}$  mice, in which a dose-dependent reduction in activated caspase labeling occurred to 18.3% and 16.7% of endplates labeled at the maximum dose of 9.6  $\mu\text{g/g}$ , compared with 48.3% and 42.8% endplates for caspase-3 and caspase-9, respectively, in the control

group ( $p < 0.001$ ). The proportions of active caspase-labeled endplates are roughly equivalent to the proportion of  $\text{Ca}^{2+}$ -overloaded endplates in untreated mice.

To detect calpain and caspase-3/-9 activities in mSCS muscle, we employed a luciferase-based luminogenic caspase substrate assay (Calpain-Glo, Caspase-9-Glo and Caspase-3-Glo, Promega) to study TA muscle homogenates. In untreated  $\epsilon\text{L269F}$  mice, calpain activity was 4.2-fold of WT, whereas caspase-3 and -9 activities were 2.7- and 2.1-fold of WT, respectively. Treatment of  $\epsilon\text{L269F}$  with fluoxetine (9.6  $\mu\text{g/g}$ ) significantly reduced calpain activity (by 2.2-fold) and caspase-3 and -9 activities by 1.3-fold, nearly to WT levels (Fig. 5B-left;  $n = 5$ ;  $p < 0.01$ ). Similarly, calpain activity in untreated  $\delta\text{S268F}$  was 5.5-fold over WT and caspase-3/-9 activities were 3.1- and 2.4-fold over WT, respectively, while after 9.6  $\mu\text{g/g}$  treatment calpain activity was reduced by 2.1-fold, and caspase-3 and -9 activities were reduced by 1.5-fold and 1.2-fold respectively, again similar to WT (Fig. 5B-right;  $n = 7$ ;  $p < 0.01$ ). These results suggest that fluoxetine-mediated reduction of  $\text{Ca}^{2+}$  overload at endplates significantly diminishes activation and accumulation of cysteine proteases, calpain, caspase-3 and -9 in mSCS muscle.

#### Fluoxetine prevents subsynaptic DNA damage in mSCS

We previously showed that subsynaptic nuclear degeneration in patients with SCS and in mSCS was associated with DNA damage indicated by TUNEL labeling or by phosphorylation of histone H2AX (pH2AX) at ser139, a histone modification that initiates DNA damage repair in association with activation of  $\text{Ca}^{2+}$ -activated degenerative enzymes (Zhu et al., 2011; Rogakou et al., 2000; Vohra et al., 2004). Based on the effect of fluoxetine on mutant AChRs,  $\text{Ca}^{2+}$  overload and protease activation in mSCS we investigated whether this treatment could reduce histological evidence of DNA damage (Zhu et al., 2011; Rogakou et al., 2000). As noted previously, immunolabeling of pH2AX was present selectively at subsynaptic nuclei of mSCS muscle, labeling approximately 37% and 45% of NMJs in both  $\epsilon\text{L269F}$  and  $\delta\text{S268F}$  mice, respectively, similar to the numbers seen for GBHA and activated caspase labeling (Fig. 5C;  $p < 0.001$ ). However, fluoxetine significantly reduced the proportion of pH2AX-labeled NMJs to  $16.1 \pm 2.8\%$  and  $37.3 \pm 2.1\%$  with 7.2  $\mu\text{g/g}$  treatment in  $\epsilon\text{L269F}$  and  $\delta\text{S268F}$  muscle, respectively, and to  $14.3 \pm 3.7\%$  and  $16.6 \pm 1.5\%$  with 9.6  $\mu\text{g/g}$  treatment, respectively (Fig. 5C;  $n = 5-7$ ;  $p < 0.001$ ). Collectively, these findings demonstrate that the drug fluoxetine, presumably because of its role as a non-specific ion channel blocker, prevents  $\text{Ca}^{2+}$  overload at NMJs through mutant AChRs, diminishes activation of cysteine proteases and reduces DNA damage in subsynaptic nuclei in SCS.



**Fig. 5.** Fluoxetine prevents pathological activation of calpain, caspase-3 and caspase-9 at NMJs, and subsynaptic DNA damage in mSCS. **A.** Left. Co-localization of activated caspase-3 (green, arrows) and caspase-9 (green, arrows) with NMJs (red) in WT and mSCS. Activated caspase-3 and caspase-9 are seen in mSCS NMJ with subsynaptic nuclei, but are not seen in WT.  $Ca^{2+}$ -mediated DNA damage in subsynaptic nuclei was exhibited as co-localization of phospho-H2AX-labeled (pH2AX, green) nuclei (DAPI, blue) with NMJ (red) in untreated mSCS.  $n = 5$ ; Scale bar = 15  $\mu m$ . Middle. Quantitation of endplates labeled with activated caspase-3 and caspase-9 in WT, untreated mSCS ( $\epsilon L269F$ ) and mSCS ( $\epsilon L269F$ ) treated with fluoxetine under indicated conditions. In untreated mSCS ( $\epsilon L269F$ ) 45.9  $\pm$  5.0% of endplates were labeled with cleaved caspase-3 and 45.2  $\pm$  5.9% with cleaved caspase-9, while no active caspases were detected in WT endplates. Treatment of mSCS ( $\epsilon L269F$ ) muscle with 9.6  $\mu g/g$  fluoxetine significantly reduced labeling for cleaved caspase-3 (18.2  $\pm$  4.1%) and caspase-9 (16.1  $\pm$  4.1%). Right. Compared with cleaved caspase-3 (48.3  $\pm$  1.8%) and cleaved caspase-9 (42.8  $\pm$  2.0%) in untreated mSCS ( $\delta S268F$ ), fluoxetine treatment with 9.6  $\mu g/g$  significantly reduced labeling for cleaved caspase-3 (21.3  $\pm$  1.8%) and caspase-9 (16.7  $\pm$  2.1%), respectively.  $n = 5$ ; \*\*\* $p < 0.001$ . All comparisons were analyzed by Student's  $t$ -test. **B.** Relative activity of calpain, caspase-3 and caspase-9 proteases (# = normalized to WT) for untreated mSCS and treated mSCS muscle under indicated conditions. Left. Protease activities for untreated mSCS ( $\epsilon L269F$ ) muscle are 4.2-fold (calpain), 2.7-fold (caspase-3), and 2.0-fold (caspase-9) to WT activity while reduced protease activities of 2.2-fold (calpain), 1.3-fold (caspase-3) and 1.3-fold (caspase-9) in  $\epsilon L269F$  treated with 9.6  $\mu g/g$  fluoxetine.  $n = 5$ ; \* $p < 0.05$ . Right. Protease activities for untreated mSCS ( $\delta S268F$ ) muscle are 5.5-fold (calpain), 3.1-fold (caspase-3), and 2.4-fold (caspase-9) to WT activity while reduced protease activities of 2.1-fold (calpain), 1.5-fold (caspase-3) and 1.2-fold (caspase-9) in mSCS ( $\delta S268F$ ) with 9.6  $\mu g/g$  fluoxetine treatment.  $n = 7$ ; \*\* $p < 0.01$ . All comparisons were analyzed by Mann–Whitney  $u$ -test. **C.** Quantitation of pH2AX-labeled NMJs in WT, untreated mSCS and mSCS treated with fluoxetine under indicated conditions. Left. In WT NMJ pH2AX-labeled nuclei were rare (1.4  $\pm$  1.2%). In untreated mSCS ( $\epsilon L269F$ ) a significant number of NMJ nuclei were labeled with pH2AX (37.3  $\pm$  2.2%). Treatment of mSCS ( $\epsilon L269F$ ) with 9.6  $\mu g/g$  fluoxetine significantly reduced pH2AX-labeling to 14.3  $\pm$  3.7%. Right. In untreated mSCS ( $\delta S268F$ ) a significant number of NMJ nuclei were labeled with pH2AX (45.1  $\pm$  2.3%). Treatment of  $\delta S268F$  with 9.6  $\mu g/g$  fluoxetine significantly reduced pH2AX-labeling to 16.6  $\pm$  1.5%.  $\epsilon L269F = 5$ ;  $\delta S268F = 7$ ; \*\*\* $p < 0.001$ . All comparisons were analyzed by Student's  $t$ -test.

**Discussion**

Detailed studies of the slow-channel syndrome and its transgenic mouse models have revealed a complex cascade of pathological events that ensue from the genetic perturbation of ion channel function, including  $Ca^{2+}$  overload, activation of cysteine proteases and DNA and organellar damage in the subsynaptic region of the NMJ. While each of these pathological steps is a potential therapeutic target, the prospect of a selective pharmacological blockade of mutant AChRs may offer the most disease-specific strategy for intervention.

In this study, we showed that fluoxetine treatment (1.5  $\mu M$ ) in vitro considerably reduces and effectively normalizes prolonged synaptic currents (MEPC decay time) in muscle of mice expressing mutant nAChRs without affecting synaptic current strength (MEPC

amplitude). This effect, seen in cultured cells also, has been attributed to the preferential blockade by open channel blockers of ion channels with prolonged open times (Harper et al., 2003). The benefit of such a pharmacological endpoint has been translated to the successful treatment of patients with SCS using fluoxetine (Harper et al., 2003; Colomer et al., 2006; Finlayson et al., 2013).

We investigated for evidence of long term, neuroprotective benefit using transgenic mice. After treating mice for 9 days mouse motor strength for mice expressing either of two SCS mutations was improved in a dose-dependent fashion as assessed using a treadmill test (Fig. 3). This clinical improvement could be attributed at least in part to improvement in neuromuscular transmission as rEMG demonstrated a dose-dependent improvement in the decremental responses in both transgenic lines (Fig. 2).

While the clinical and electrophysiological findings demonstrate improvement of neuromuscular function in experimental SCS, supporting clinical experience, they do not provide evidence for long-term benefit in SCS, a degenerative disease of the NMJ. Because the principal mediator of the degenerative process appears to be elevated  $\text{Ca}^{2+}$ , entering from the extracellular space through mutant AChRs, and from internal stores through the  $\text{IP}_3\text{R}_1$  when activated by the binding of  $\text{Ca}^{2+}$  and  $\text{IP}_3$  (Gomez et al., 2002a; Zhu et al., 2011), we investigated the ability of fluoxetine to reduce subsynaptic  $\text{Ca}^{2+}$  overload. Fluoxetine had a dose-dependent reduction in the proportion of NMJs in which histological evidence of  $\text{Ca}^{2+}$  could be detected in mice expressing either SCS mutation (Fig. 4). Furthermore, because  $\text{Ca}^{2+}$  is implicated in the activation of downstream proteolytic enzymes in degenerative processes, and there is pronounced activation of cysteine proteases in SCS muscle (Zhu et al., 2011, 2013, 2014; Groshong et al., 2007; Vohra et al., 2004, 2006), we tested whether the reduction in endplate  $\text{Ca}^{2+}$  overload following fluoxetine treatment was accompanied by changes in the elevated activity of proteases. Fluoxetine treatment markedly decreased postsynaptic caspase-3 and -9 activities, and the tissue activities of both these caspases as well as calpain in both transgenic lines in a dose-dependent fashion (Fig. 5). Finally, the endpoint of the degenerative process in SCS is a pronounced organellar damage, leading to an appearance of a localized apoptotic process affecting mitochondria and nuclei. In SCS and its mouse model subsynaptic nuclei in the NMJ are labeled using the TUNEL assay as well as for phosphorylated H2AX, a marker of double stranded DNA damage (Zhu et al., 2013, 2014; Vohra et al., 2006; Rogakou et al., 2000). We found that after nine days of treatment of mSCS mice with increasing doses of fluoxetine there was a dose-dependent reduction in the proportion of subsynaptic nuclei in the NMJ demonstrating DNA damage (Fig. 5).

These findings demonstrate that fluoxetine treatment provides not only for functional improvement, but dramatically reduces the biochemical and cellular measures of degeneration in the neuromuscular system in SCS, consistent with a mechanistic basis of pharmacological correction the genetic perturbation of AChR gating and prevention of the downstream consequences of cationic overload. These results suggest that SCS patients will achieve neuroprotective benefit from long-term with fluoxetine or other open ion channel blockers.

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