Selective inhibition of caspases in skeletal muscle reverses the apoptotic synaptic degeneration in slow channel myasthenic syndrome

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ABSTRACT

The slow-channel syndrome (SCS) is a congenital myasthenic disorder caused by point mutations in subunits of skeletal muscle acetylcholine receptor (AChR) leading to Ca$^{2+}$ overload and degeneration of the postsynaptic membrane, nuclei, and mitochondria of the neuromuscular junction (NMJ). In both SCS muscle biopsies and in transgenic mouse models for SCS (mSCS), the endplate regions are shrunken, and there is evidence of DNA damage in the subsynaptic region. Activated caspases-9, -3, and -7 are intensely co-localized at the NMJ, and the Ca$^{2+}$-activated protease, calpain, and the atypical cyclin-dependent kinase, Cdk5, are over-activated in mSCS. Thus, the true mediator(s) of the disease process are not clear. Here, we demonstrate that selective inhibition of effector caspases, caspase-3 and -7, or initiator caspase, caspase-9, in limb muscle in vivo by localized expression of recombinant inhibitor proteins dramatically decreases subsynaptic DNA damage, increases endplate area and improves ultrastructural abnormalities in SCS transgenic mice. Calpain and Cdk5 are not affected by this treatment. On the other hand inhibition of Cdk5 by expression of a dominant negative form of Cdk5 has no effect on the degeneration. Together with previous studies these results indicate that, focal activation of caspase activity at the NMJ is the principal pathological process responsible for the synaptic apoptosis in the SCS. Thus, treatments that reduce muscle caspase activity are likely to be of benefit for SCS patients.
INTRODUCTION

The slow-channel syndrome (SCS) is a dominantly-inherited congenital myasthenic syndrome, caused by gain-of-function, missense mutations in any of the four subunits of the acetylcholine receptor (AChR) (1,2). The mutant AChRs in SCS exhibit delayed channel closure, leading to Ca\(^{2+}\) overload and degenerative changes of the postsynaptic region of the neuromuscular junction (NMJ), impaired synaptic transmission, and progressive muscle weakness (1,3). Degenerative changes consist of a widened, debris-laden synaptic cleft, degenerating nuclei and mitochondria, and DNA damage, and localized activation of initiator (caspase-9) and effector caspases (caspase-3, -7) resembling a localized form of apoptosis (4-6). Because the SCS represents a prototype disease of synaptic degeneration, we have developed and extensively characterized mouse models for SCS (mSCS) by targeting expression of AChR subunits bearing mutations found in the human disease (3,5). The models exhibit all the clinical, electrophysiological, and pathological features of the disease, and have allowed pharmacological and genetic manipulation to dissect the disease pathways and explore therapies (7-10). Examination of mSCS muscle has demonstrated extensive activation of three dominant enzyme systems involved in neurodegeneration, the caspases, calpain and the atypical cyclin-dependent kinase, type 5 (Cdk5). Elimination of Ca\(^{2+}\) overload by prevention of internal Ca\(^{2+}\) release in mSCS muscle leads to reduced activation of calpain and caspase 3/9, and improvement of subsynaptic DNA damage and neuromuscular transmission, but until now there has been no clear idea of which enzymes are responsible (6,9,10).

The caspase proteases are a family of cysteine proteases that participate in an activation cascade commonly identified as responsible for direct mediation of critical steps leading to apoptosis, due to the large number of cellular caspase substrates (11). However, the caspases, including the effector or executioner caspases, caspase-3 and -7 once thought to be the key mediators of cell death in apoptosis and the point of no return, now have also been associated with non-death functions such as neuroprotection, synaptic remodeling, activation of microglia (12-14). Caspases are essential for survival and their experimental inhibition often only alters morphological features of death without preventing cell death (15,16).
Calpain is a Ca\(^{2+}\)-activated protease involved in a wide array of physiological and pathological proteolytic events including membrane fusion, growth cone extension, intracellular signaling, and excitotoxicity (17-20). Calpain also dysregulates the atypical cyclin-dependent kinase, type 5 (Cdk5) by the cleavage of p35 to p25, a co-activator of Cdk5, which mediate DNA damage signaling and regulate neuronal death (19,21-24).

Transgenic expression of calpastatin, an endogenous inhibitor of calpain, in mSCS improves strength and neuromuscular transmission, but does not reduce synaptic apoptosis or caspase activation at NMJs (9).

In this study we explored further the pathways involved in the post-synaptic degeneration at the NMJ in the SCS by their selective blockade in mSCS. We show that expression of a dominant-negative form of caspase 9, (C9DN) or the protein, human apoptosis inhibitor 4 (API4/survivin), an endogenous broad-spectrum caspase inhibitor, in TA muscle markedly reduced caspase-9 and -3 activities and diminished subsynaptic DNA damage in mSCS. Specific inhibition of caspase-9 and -3 activities significantly improved endplate area and rescued ultrastructural changes in mSCS muscle. In comparison, inhibition of cyclin-dependent kinase, type 5 (Cdk5) activity in muscle had no effect on DNA damage or on ultrastructural changes in mSCS muscle, nor did it attenuate caspase-9 and -3 activities. These findings demonstrate that focal activation of caspase-9 and -3 at NMJs, but not Cdk5-ATM pathway, is essentially responsible for subsynaptic DNA damage and apoptotic synaptic degeneration in SCS disease.

RESULTS

Expression of recombinant API4 protein is accumulated in subsynaptic nuclei of mSCS muscle, but not in WT. To transiently inhibit caspases in mSCS muscle we directly electroporated plasmid vectors expressing recombinant API4 into limb muscle (25). Nine days after electroporation of pcDNA3-API4 into TA muscle, we detected abundant levels of API4 protein in WT and mSCS. No API4 was detected in the contralateral TA muscle treated with pcDNA3 as control, nor in untreated WT or mSCS adult muscle in agreement with previous studies (Figure 1A) (25,26). Using specific
antibody to API4 we detected immunostaining for the protein distributed around myonuclei in WT muscle. Anti-API4 antibody labeled most myonuclei, but was extensively co-localized with subsynaptic nuclei in mSCS muscle (Figure 1B). The number of API4-labeled endplates in API4-treated mSCS muscle (42.2±4.9%) was 7-fold greater than in API4-treated WT (6.3±0.5%; Figure 1B; n=7; p<0.001). Extensive accumulation of recombinant API4 in subsynaptic nuclei correlates with the presence of activated caspases-3, -7, and -9 in the SCS muscle and is consistent with the capacity of API4 to bind active caspases (6,9,10,27-29).

**Inhibition of caspase-3 and caspase-9 activity in mSCS muscle.**

As shown previously immunolabeling with antibodies specific for cleaved caspase-3 and -9 demonstrates that activated caspase-3 and caspase-9 are present at a significant proportion of SCS NMJs (Figure 2A, left) (6,10). Expression of API4 in mSCS TA muscle reduced the proportion of activated caspase-3 at NMJs by 3-fold, (45.9±5.0% vs 13.7±3.8%, p< 0.001) and the proportion of activated caspase-9 at NMJs by nearly 2-fold, (45.2±5.7% vs 24.9±3.8%, p< 0.001; Figure 2A, right). Similarly, treatment of mSCS muscle with C9DN plasmid reduced the proportion of activated caspase-9 at NMJs by nearly 3 fold, (45.2±5.7% vs 16.1±2.3%; n=5; p<0.001), and the proportion of activated caspase-3 at NMJs by nearly 2-fold (45.9±5.0% vs 24.1±2.6%; n=5; p<0.001; Figure 2A, right). These results suggest that reduction of caspase-3 and -9 activities in the SCS muscle dramatically decreases activated caspase-3 and -9 expression in NMJs.

To further investigate the effect of inhibition of API4 and C9DN on caspase-3 and -9, we employed a sensitive luminescence assay to measure caspase-3 and -9 activities in API4- or C9DN-electroporated mSCS (10,30). In untreated mSCS muscle, caspase-3 and -9 activities were 3-fold and more than 2-fold greater than WT, respectively (n=5; p<0.01; Figure 2B), as shown previously (10). In muscle expressing API4, the activities of caspase-3 and -9 were reduced to 1.2 and 1.5 of untreated respectively, compared with 2.7 and 2.2 in control plasmid (p<0.01). Expression of C9DN reduced caspase-3 and -9 activities to 1.8 and 0.8 of untreated mSCS muscle, respectively (n=5; p<0.01;
Figure 2B). There was no difference between untreated mSCS and mSCS treated with control plasmid (n=5; p>0.95).

Endplates of both SCS patients and mice show overloading with Ca\(^{2+}\) due to disturbed AChR channel gating (6,10). To exclude an effect of API4 or C9DN on endplate Ca\(^{2+}\) overload we used the histochemical stains, GBHA, to detect Ca\(^{2+}\) and acetylcholinesterase in serial sections to localize NMJs and determine proportion of endplates with detectable Ca\(^{2+}\) overload (31,32). API4- and C9DN-electroporated mSCS muscles showed similar numbers of Ca\(^{2+}\) overloaded endplates as in untreated mSCS muscle (Figure 2C, left). GBHA-labeled endplates were 35.7±3.4% in pcDNA3-treated mSCS vs 36.7±3.1% in API4-treated mSCS muscle and vs 40.2±6.7% in C9DN treated muscle (p>0.95), compared with no GBHA staining in WT (n=5; p<0.001; Figure 2C, right). These results demonstrate that expression of recombinant API4 and C9DN specifically diminishes caspase-3 and -9 activities in SCS muscle without affecting the marked Ca\(^{2+}\) overload at endplates.

Inhibition of caspase 3/9 activity reduces DNA damage in mSCS.
Phosphorylation of the histone protein, γH2AX (p-H2AX), has been used as a marker of DNA double strand breaks (33). As shown previously immunostaining with antibody specific to p-H2AX labels a significant proportion of subsynaptic nuclei in mSCS compared with rare nuclei labeled in WT muscle, suggesting that subsynaptic nuclei undergo double-stranded DNA damage in mSCS (Figure 2D, upper) (10). Treatment of mSCS muscle by expression of API4 reduced the proportion of p-H2AX-labeled subsynaptic nuclei by more than 2-fold, (15.8±2.0% vs 37.3±2.2%; p<0.001; Figure 2D, lower). C9DN reduced p-H2AX-labeled NMJs to 25.7±0.7%, compared with 35.6±3.3% in mSCS control (p<0.001; Figure 2D, lower). These results suggest that inhibition of caspase-3 and -9 activities diminishes subsynaptic DNA damage in SCS muscle.
Inactivation of caspase-3 and -9 activities increases endplate area and improves ultrastructure in SCS mouse. SCS is associated with reduced endplate area, AChR density and degeneration of postsynaptic structures. To investigate the effects of caspase inhibition on endplate area in mSCS muscle, we used neurofilament antibody and Txr-αBT to visualize endplate structure. Untreated mSCS NMJs are small and shrunken compared with WT (Figure 3A, left and middle). Nine days after expression of API4 mSCS NMJs appeared larger than untreated muscle. Treatment with C9DN caused a similar effect (Figure 3A, left and middle). Caspase inhibition in mSCS muscle also increased endplate size. Quantitation of endplate size (ImageJ software) showed that the mean area of mSCS endplates was less than one half that of WT endplates (259.55±46.11 vs 598.36±74.13 vs), while API4-treated mSCS were 1.8 fold that of mSCS (460.09±48.95 vs 259.55±46.11), and C9DN-treated mSCS were 1.6 fold that of mSCS (429.11.36±79.22 vs 259.55±46.11; p<0.001; Figure 3A, right-lower). No difference was noted between mSCS untreated and mSCS control (mSCS-pcDNA3; 259.55±46.11 vs 263.16±61.23; p>0.97). The AChR densities (defined as pixel number per endplate) paralleled the changes in endplate area and was also significantly increased by either treatment, compared with control (Figure 3B, right-upper; p<0.01).

We used electron microscopy to examine the ultrastructural alterations in WT, mSCS and treated mSCS muscles. As noted previously untreated mSCS NMJs had poorly identified nerve terminus, degenerating subsynaptic nuclei and mitochondria, paucity of postsynaptic folds and presence of debris in the synaptic cleft (3,7,8). Degenerating subsynaptic nuclei, the most consistent and quantifiable change in SCS, may reflect the changes seen in p-H2AX labeling. The proportion of subsynaptic nuclei that are degenerating is shown in Figure 3B (left). In mSCS muscle treated with API4, NMJs showed an obvious improvement. Of 26 NMJs examined 25 showed normal subsynaptic nuclei with intact postsynaptic clefts, except for slightly abnormal nerve terminus. These changes were statistically significant based on chi-square analysis (Figure 3B, right; p<0.05). The changes were also present but less apparent in C9DN-treated NMJs.
**Amplified Cdk5 activity is not involved in subsynaptic DNA damage in mSCS.** Cdk5 is part of a prominent signaling pathway in neural cells, which is activated in neurodegenerative diseases (24, 34, 35). Activated Cdk5 phosphorylates the ataxia-telangiectasia mutated protein (ATM) in response to DNA damage, which regulates DNA damage-induced neuronal death (24). To identify Cdk5 expression in WT, mSCS and treated mSCS muscles, we used specific Cdk5 antibody in immunolocalization studies. We found that Cdk5 co-localized with NMJs and surrounding subsynaptic nuclei to a similar degree in both WT and mSCS (Figure 4, left). Cdk5 activity is elevated in mSCS muscle, as measured by a H1 phosphorylation assay (21). To reduce Cdk5 activity in muscle we electroporated TA muscles with a plasmid vector expressing Cdk5DN and compared homogenates of these muscles with those electroporated with control plasmid. 9 days after electroporation of Cdk5DN, the activity of Cdk5 was reduced to 30% of untreated mSCS muscle (0.63±0.13 vs 2.2±0.3; p<0.05; Figure 4, right-lower) while control treated mSCS muscle had similar activity to untreated mSCS (2.2±0.2 vs 2.2±0.3; p>0.98; Figure 4, right lower). Neither API4 nor C9DN treatment had an effect on Cdk5 activity in mSCS muscle (p>0.98; Figure 4, right-upper). These results showed that Cdk5 activity was amplified in mSCS and can be reduced by expression of the dominant negative form of Cdk5.

To investigate the role of dysregulated Cdk5 activity in the subsynaptic DNA damage in mSCS muscle we measured the number of p-H2AX-labeled NMJs in mSCS muscle transfected with Cdk5DN vs control plasmid. 9 days after Cdk5DN treatment, there was no difference in the number of p-H2AX-labeled NMJs in Cdk5DN-treated mSCS muscle (35.7±2.0%) compared with untreated muscle (37.3±2.2%; n=5; p>0.98; Figure 4B), while only 1.4 ± 1.2% were labeled with p-H2AX in WT muscle (n=5; p<0.001). These results suggest that increased Cdk5 activity does not play a role in subsynaptic DNA damage in SCS muscle or p-H2AX phosphorylation at the NMJ.
Discussion

The slow-channel syndrome represents a prototype disease of synaptic degeneration, and has been ideal model disease for delineating the in vivo molecular and cellular pathways, as well as the ultrastructural changes, involved in an ongoing, excitotoxic, degenerative process mediated by synaptic Ca\(^{2+}\)-overload. While the NMJ is a highly specialized excitatory synapse, most of the components involved in the synaptic function of the NMJ are present in CNS excitatory synapses that are not accessible to this level of analysis. Thus these findings are relevant to CNS synaptic disorders.

Having previously excluded direct involvement of activated calpain in the postsynaptic degenerative process, in the present study we explored two additional pathways associated with neurodegenerative disease and apoptotic cell death in cultured cells. Using localized expression of recombinant inhibitor proteins in limb muscle by electroporation of plasmid expression vectors we found that, components of the intrinsic caspase pathway play the predominant role in the localized organellar degeneration in mSCS, while pronounced activation of the atypical cyclin-dependent kinase, Cdk5, does not appear to contribute to the disease process in the postsynaptic region. Both the widespread caspase inhibitor, ApI4 (a broad spectrum endogenous caspase inhibitor that targets caspase-3 and -7) and a dominant negative form of caspase-9 expressed in TA muscle reduced the signs of DNA damage, improved endplate size, AChR density and reduced ultrastructural evidence of organellar damage in NMJs within nine days of expression. In addition, the activities of caspase-3 and -9, and NMJs labeled with activated caspase -3 and -9 in ApI4-expressed SCS muscle are still higher than in normal WT muscle, which demonstrates that expression of ApI4 is predominantly relevant to prevention of overactivated caspases in SCS muscle.

Caspase inhibition in mSCS reversed degenerative changes despite the ongoing overload of endplates with excessive Ca\(^{2+}\), the presumed trigger of the caspase activation. Activated calpain also persisted in the mSCS muscles after caspase inhibition, further dissociating calpain from a role in the degenerative process. This was previously suggested by the calpastatin transgenic studies that normalized calpain activity in mSCS muscle without affecting endplate degeneration. Thus, the
caspase enzymes play a key role in the degenerative process mediated excessive Ca^{2+} in the mSCS, and by extrapolation, in SCS. Their blockade results in the reversal of key structural abnormalities that impair endplate function and neuromuscular transmission.

In contrast to the effect of caspase inhibition, blockade of Cdk5 activity by expressing a dominant negative form of the enzyme had no effect on DNA damage markers, endplate area, or ultrastructural abnormalities. Thus, while Cdk5 has been implicated as mediator of DNA-damage induced cell death in some models, this study argues against either a direct role in the degenerative process or even eliciting the DNA damage response histone modifications. We show in other studies that Cdk5 appears to play a regulatory role for CaM Kinase activation and nNOS signaling that provides a retrograde inhibitory signal mediated by NO (36) (Figure 5).

Although the caspases have been widely implicated in disease by being the key proteases underlying apoptotic cell death, as well as playing a role in neurodegenerative diseases, there is little direct evidence for a causal role for these cysteine proteases in human neurological disease (37-40). Moreover, the caspases have also been associated with several non-death functions such as neuroprotection, synaptic remodeling, activation of microglia (12-14). We previously showed in transgenic SCS mice and human muscle biopsy specimens from SCS patients that activated caspases-9, -3, and -7 are intensely co-localized at the neuromuscular junction in SCS but not in normal muscle, although we have seen here that other mediators of degeneration are present. The present study uses in vivo transfection to selectively eliminate each of the potential mediators to identify caspase-9 and caspase-3/7 effectors of the degenerative process. Thus, this study provides strong evidence that localized activation of caspases at the neuromuscular junction are responsible for an ongoing focal degenerative process resembling localized apoptosis. It suggests that treatments for SCS will be successful as long term therapies if they reduce caspase activation. These studies also suggest that caspase activation may play a role in disorders thought to be mediated by excitotoxic damage, such as ALS, HD, stroke, and epilepsy.
MATERIAL AND METHODS

Materials

Chemical reagents were purchased from Sigma Chemical Co. Cell culture materials were obtained from Gibco BRL Co. Laminin, alpha-bungarotoxin (αBT) conjugated Texas Red (TxR-αBT), fluorescence-tagged secondary antibody 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: API4 (1:1000; SCBT), ataxia telangiectasia mutated kinase (ATM; 1:1000; Millipore), phosphorylated ATM at Ser 1981 (p-ATM (1981); 1:1000; Cell Signaling), phosphorylated H2AX at Ser139 (p-H2AX; 1:500; Millipore), cleaved caspase 3 and 9 (1:200; Cell Signaling), Cyclin-dependent kinase 5 (Cdk5; 1:500; SCBT), p35/p25 (1:1000; Cell Signaling) and glyceraldehyse 3-phosphate dehydrogenase (GAPDH; 1:5000; Ambion).

Plasmid and construct assembly

The pcDNA3 plasmid expressing API4 under the control of CMV promoter was kindly supplied by Dr. Dario C. Altieri (25). Dominant negative caspase 9 (pIRES-EGFP-casp9DN), generous gift of Dr. Scott Lowe (41), was cloned into pcDNA3 plasmid (C9DN). Dominant negative Cdk5 plasmid (Cdk5DN; K33T) cloned into pcDNA3 was a gift of Dr. H. Sinichi (42).

Animals and tissue preparation

4-6 month-old male wild type FVB mice and SCS transgenic mice (εL269F; mSCS) were used in this study (7). All 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 (43).

Western Blotting

Tissue was homogenized in 50mM Tri-HCl, pH 7.6, 150mM NaCl, 1% SDS, 1% Triton X-100, 0.1mM EGTA, and 0.1mM EDTA supplemented with protease inhibitor cocktail (Roche).
Homogenates were fractionated by SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-
rad, Hercules, U S). Membranes were blocked for 1hr in TBS-T (50 mM Tris.HCl, pH 7.4, 150 mM
NaCl and 0.05% Tween-20) containing 5% non-fat milk, and then incubated for overnight with primary
antibodies. After 3-time wash in TBS-T, membrane was incubated with HRP-conjugated secondary
antibody for 1 hr and washed 3x. Immunolabeled bands were developed using ECL reagents (Ambion,
Inc.) on X-ray film. Reaction product levels were quantified by scanning densitometry using Image J
(5.3 version).

Tissue Staining and Imaging

Ca\(^{2+}\)-overloaded endplates were detected in cryosections using histochemical stains for
cholinesterase and Ca\(^{2+}\) (GBHA, glyoxal bis 2 hydroxyanil) as described (31,32).
Immunohistochemistry and confocal fluorescence microscopy was detailed as previously (10).

Quantitative studies in tissue sections

Quantitation of the proportion of labeled NMJs in tibialis muscle (TA) cryosections was carried out
using sequential alternate sections stained for cholinesterase and test label (GBHA, p-H2X and
caspase 3/9) as described previously (10), employing Image J (v5.3).

Calpain and caspase activity assay

Calpain, caspase-3 and caspase-9 activities in muscle or cell homogenates were measured using
a firefly luciferase-based assay (Calpain-Glo™ Protease Assay; Caspase-Glo® 3/7 Assay; Caspase-
Glo® 9 Assay; Promega). Cell and muscle tissue were homogenized as described previously (44)
except for the addition of 10mM NH\(_4\)Cl and 10mM 3-methyladenine to the homogenization buffer to
stabilize lysosomes and proteosome. Protein samples (20µg) were analyzed by luminometer (Turner
BioSystems, Inc) in triplicate.
Cdk5 kinase assay

Tissue was homogenized with lysis buffer. The supernatants (500 µg) were then collected and immunoprecipitated with Cdk5 antibody (5µg). The Cdk5 immunoprecipitates were washed twice with the lysis buffer and twice with a kinase buffer containing 50 mM Tris pH 7.5, 5 mM MgCl$_2$, 1mM EGTA, 1mM EDTA, 40 mM β-glycerophosphate, 20 mM p-nitrophenylphosphate, 0.1 mM sodium vanadate and 1 mM DTT. Kinase activity was measured in a total volume of 50 µl of kinase assay buffer with 0.1mM [$\gamma$-$^{32}$P]ATP (PerkinElmer) and 10 µg histone H1 (Calbiochem) at 30 °C for 60 min. The reaction was stopped by adding SDS sample loading buffer (4X). Samples were separated by SDS-PAGE gel (15%), transferred to nitrocellulose membrane, and autoradiographed to detect phosphorylation of histone H1. Cdk5 activity in different tissue samples was normalized via the ratio of phosphorylated histone H1 to internal Cdk5 expression.

Intramuscular electroporation-mediated gene delivery

Electroporation of plasmid was carried out as described (45). Briefly, after anesthesia hind limbs were shaved and TA muscles were injected with hyaluronidase (30 µl; Sigma-Aldrich,) 2hr prior to performing electroporation. Subsequently, 10µg expression plasmids in saline were injected into TA muscle. Immediately following injection, 10 separate electrical pulses (10ms) were delivered through circle electrodes (1cm-diameter) applied to ventral and dorsal surfaces flanking the TA at 175 V/cm with 1 Hz via a square-wave electroporator (ECM830, Harvard Apparatus, Holliston, MA). Control side was similarly electroporated with pcDNA3 plasmid.

Electron Microscopy

The procedures of tissue fixation, section and embedding and manipulation of EM equipment were described as before (7). Five TA muscles electroporated with the expression plasmid for dominant negative caspase 9, five with the API4 plasmid for and 10 controls from the contralateral TAs electroporated with control plasmid (pcDNA3) were examined. The number of degenerate apoptotic nuclei was quantified in each group. 114 postsynaptic nuclei from 77 neuromuscular junctions were
examined for the controls. 44 postsynaptic nuclei from 26 neuromuscular junctions were examined for the API4 treated muscles, 33 postsynaptic nuclei from 24 neuromuscular junctions were examined for the caspase 9 dominant negative samples.

**Statistics**

Values were represented as the mean ± 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.
References


Figure Legends

Figure 1. Expression of recombinant API4 in TA muscle by electroporation

A. **Upper:** 9-days after electroporation of API4 plasmid in WT and mSCS TA muscle there was strong expression API4 protein in TA western blots. No API4 protein was detected in WT, mSCS and mSCS with electroporation of pcDNA3 as control (mSCS-pcDNA3). **Lower:** Relative protein levels of API4 (# = normalized to GAPDH) in API4-electroporated WT (WT-API4) and mSCS (mSCS-API4) TA muscle (0.79±0.03 and 0.74±0.05, respectively) were significantly greater than the undetectable levels in untransfected muscle. n=3; ***p<0.001, Student’s *t*-test.

B. **Upper:** API4, immunostained with antibody to API4 (green) was not co-localized with AChRs labeled with TxB-RαBT (red) but predominantly surrounded subsynaptic DAPI-labeled nuclei (blue) in API4-electroporated WT TA muscle (WT-API4). However, API4 labeling was much more prominent in subsynaptic nuclei in API4-transfected mSCS muscle (mSCS-API4) than WT muscle. **Lower:** 42.2±4.9% of endplates labeled for API4 in mSCS-API4 mice, while compared with 6.3±0.5% in WT-API4 mice. Scale bar=15μm; n=7; ***p<0.001, Student’s *t*-test.

Figure 2. Inhibition of caspase 3/9 activity in TA muscle reduces DNA damage in subsynaptic nuclei in mSCS

A. **Left:** Intense labeling of untreated NMJs (red) in mSCS muscle with antibody to C3 (green, arrows) and C9 (green, arrows), was decreased or completely eliminated in mSCS muscle expressing API4. **Right:** In mSCS-API4 muscle 13.7±3.8% of endplates were labeled with anti-C3 and 24.9±3.8% with anti-C9, compared with 45.9±5.0% for C3 and 45.2±5.7% for C9 in pcDNA3 treated mSCS. In mSCS muscle transfected with C9DN (SCS-C9DN) 24.1±2.6% of endplates were labeled with C3 and 16.1±2.3% with C9, compared with 43.9±4.9% for C3 and 46.3±6.4% for C9 in mSCS-pcDNA3. Scale bar=15μm; n=5; ***p<0.001, Student’s *t*-test.
B. Relative protease activity of caspase-3 (C3) and caspase-9 (C9) (# = normalized to WT) in WT, mSCS and treated mSCS. In mSCS-API4, C3 was 1.20±0.2 of WT and C9 was 1.5±0.2 of WT, compared with 3.0±0.5 for C3 and 2.3±0.2 for C9 in mSCS. Caspase-3 and -9 activities in mSCS-C9DN were reduced to 1.8±0.3 and 0.8±0.2, respectively, compared with 2.8±0.4 for C3 and 2.2±0.2 for C9 in mSCS-pcDNA3. n=5; **p<0.01, Mann-Whitney u-test.

C. Left: Serial TA muscle sections labeled for cholinesterase (dark brown) to localize endplates and glyoxal bis-2-hydroxyanil (GBHA) (red) to localize Ca²⁺ overloading in WT and mSCS muscle. The numbers on GBHA-labeled fibers correspond to fibers with cholinesterase-stained endplates in adjacent section. Ca²⁺-overloaded endplates were found in mSCS muscle but not in WT. Right: Quantitation of Ca²⁺-overloaded endplates showed that no GBHA-stained endplates were found in WT compared with 37.8±4.3% GBHA-stained endplates in mSCS muscle, 36.7±3.1% in API4-expressing mSCS muscle (SCS-API4), 40.2±6.7% in C9DN-expressing mSCS muscle (SCS-C9DN), 38.5±5.8% in Cdk5DN SCS-Cdk5DN), and 35.7±3.4% in SCS control (SCS-pcDNA3). n=5; p>0.95.

D. Upper: API4 prevents subsynaptic DNA damage in mSCS muscle. phospho-γH2AX-labeled (p-H2AX, green) nuclei (DAPI, blue) were co-localized with NMJ (red) in mSCS muscle, but significantly diminished in subsynaptic nuclei in mSCS-API4. Lower: 15.8±2.0% of endplates were labeled with p-H2AX in mSCS-API4 compared with 37.3±2.2% in mSCS and 35.6±3.3% in mSCS control (SCS-pcDNA3). 25.7±0.7% of endplates in mSCS-C9DN were labeled with p-H2AX. Only 1.42±1.23% of WT endplates were labeled with p-H2AX. Scale bar=15μm; n=5; ***p<0.001, Student’s t-test.
Figure. 3 Inhibition of caspase 3 and 9 activities increases AChR density and improves ultrastructural changes of NMJ in mSCS.

A. Left. Neuromuscular junctions of WT, mSCS, and mSCS treated with API4, or C9DN immunolabeled with neurofilament-specific antibody (green) and TxA-αBT (red) in WT, mSCS and treated mSCS muscle. Scale bar=25µm; n=5. Scale bar=25µm; n=5. **Middle:** Images from A showing only AChR labeling with TxA-αBT for AChR density area measurements. **Right. Upper:** AChR density in WT, mSCS and treated mSCS (# = normalize to WT). Electroporation of AIP4 and C9DN to mSCS muscle increased AChR density to 0.78±0.05 and 0.85±0.06 of WT, respectively, whereas 0.49±0.02 in mSCS and 0.51±0.03 in mSCS control (SCS-pcDNA3; **p<0.01, Mann-Whitney u-test). **Lower:** Endplate area (µm²) in WT, mSCS and treated mSCS. Endplate area was 598.36±74.13 in WT, 460.09±48.95 in SCS-API4 and 429.11±79.22 in mSCS-C9DN, compared with 259.55±46.11 in mSCS and 263.16±61.23 in mSCS control (SCS-pcDNA3). n=5; ***p<0.001, Student’s t-test. Number of end plates=50/sample.

B. Ultrastructure of NMJs from WT, untreated control mSCS and treated mSCS muscle. NMJs of mSCS muscles exhibited ultrastructural changes as previously described (left). Degenerative changes in post-synaptic nuclei were improved in the treated mSCS muscles. The number of degenerative nuclei with apoptosis-like changes was quantified (right). 20.18% (23 of 114) of junctional nuclei in mSCS muscles showed degenerative changes (left, bottom row). WT muscle with normal subsynaptic nucleus is shown for comparison. API4 treated mSCS muscle showed improvement in ultrastructural abnormalities with a significant decrease in the number of degenerative nuclei to 2.27% (1 of 44). A similar trend was seen in the C9DN treated mSCS animals with 9.09% degenerative nuclei (3 of 33). This trend did not reach statistical significance. The size bars correspond to 2 µm. The p-values for the comparison between control mSCS muscles and API4 treated mSCS muscles or between control muscles and C9DN treated mSCS muscles respectively were calculated by Chi-square testing. *p<0.005; *p<0.14.
Figure 4. Reduction of Cdk5 activity does not improve subsynaptic DNA damage in mSCS muscle.

A. **Upper:** Immunostaining of Cdk5 (green) co-localized with NMJ (red) in WT and mSCS. No morphological changes were identified between WT, mSCS and treated mSCS TA muscle. **Middle:** Cdk5 activity is increased in mSCS muscle compared with WT. Electroporation of Cdk5DN plasmid reduced Cdk5-dependent phosphorylated Histone H1 level in mSCS muscle whereas API4 expression had no effect on Cdk5 activity. **Lower:** Quantification of Cdk5 activity in WT, mSCS and treated mSCS TA muscle expressed as the ratio of phosphorylated histone H1 level to Cdk5 protein expression level (# = normalized to WT). In mSCS-Cdk5DN, Cdk5 activity was 0.63±0.13 of WT, compared with 2.2±0.3 in SCS, 2.2±0.1 in mSCS-API4 and 2.2±0.2 in mSCS-pcDNA3. n=3; *p<0.05, Mann-Whitney u-test.

B. Quantitation of p-H2AX-labeled endplates in WT, mSCS and treated mSCS. p-H2AX-labeled endplates in mSCS-Cdk5DN was 35.7±2.0%, compared with 37.3±2.2% in mSCS and 35.6±3.3% in mSCS control (mSCS-pcDNA3). n=5; p>0.98. p-H2AX-labeled endplates in WT was 1.42±1.23%. n=5; ***p<0.001, Student’s t-test.

Figure 5 Diagram of molecular mechanism of SCS disease

Prolonged openings of mutant AChRs in SCS allow entry of excess Ca^{2+}, which combines with IP3 (inositol 1, 4, 5-triphosphate) to activate IP3R1 (type I inositol 1, 4, 5-triphosphate receptor) causing further Ca^{2+} release from internal stores (Ref.10). Excessive sarcoplasmic Ca^{2+} activates caspase-3 and -9 as well as calpain at postsynaptic area. Activated caspase-3 and 9 enter into subsynaptic nuclei leading to DNA damage. Inhibition of caspase-3 with API4 reduces subsynaptic DNA damage and ultrastructural evidence of subsynaptic nuclear degeneration. Similarly, inhibition of caspase-9 activity using dominant negative caspase-9 (C9DN) reduces subsynaptic DNA damage and subsynaptic nuclear degeneration. As we previously showed, activated calpain at the postsynaptic area in SCS has a predominantly presynaptic action, through activation of Cdk5 by...
cleavage of p35, Cdk5 co-activator, and increased NO production via nNOS (neural nitric oxide synthase) pathway (Ref. 9, 41). Here, our studies demonstrate that neither activated calpain nor increased Cdk5 activity are not involved in subsynaptic DNA damage.