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Calpain activation impairs neuromuscular transmission in a mouse model of the slow-channel myasthenic syndrome

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The slow-channel myasthenic syndrome (SCS) is a hereditary disorder of the acetylcholine receptor (AChR) of the neuromuscular junction (NMJ) that leads to prolonged AChR channel opening, Ca2+ overload, and degeneration of the NMJ. We used an SCS transgenic mouse model to investigate the role of the calcium-activated protease calpain in the pathogenesis of synaptic dysfunction in SCS. Cleavage of a fluorogenic calpain substrate was increased at the NMJ of dissociated muscle fibers. Inhibition of calpain using a calpastatin (CS) transgene improved strength and neuromuscular transmission. CS caused a 2-fold increase in the frequency of miniature endplate currents (MEPCs) and an increase in NMJ size, but MEPC amplitudes remained reduced. Persistent degeneration of the NMJ was associated with localized activation of the non-calpain protease caspase-3. This study suggests that calpain may act presynaptically to impair NMJ function in SCS but further reveals a role for other cysteine proteases whose inhibition may be of additional therapeutic benefit in SCS and other excitotoxic disorders.

Introduction
The slow-channel congenital myasthenic syndrome (SCS) is a dominantly inherited neuromuscular disorder characterized by impaired neuromuscular transmission and degeneration of the neuromuscular junction (NMJ) (1–3). Patients with SCS manifest fatigability and progressive weakness of the skeletal muscles, with symptoms ranging from eye muscle and mild limb weakness to severe incapacitation and respiratory failure. Ultrastructural study of muscle in SCS reveals a focal degenerative and remodeling process selectively localized to the NMJ termed endplate myopathy. This progressive, purely synaptic disease process, in which the cleft is widened and nerve terminals are shrunken, is characterized by expansion and degeneration of postsynaptic folds as well as degeneration of subsynaptic nuclei, mitochondria, and myofibrils (1–4).

The SCS is caused by missense mutations within the genes encoding nicotinic acetylcholine receptor (AChR) subunits. The mutations alter the AChR channel gating function, causing prolonged activation events, persistent synaptic currents, and localized calcium overload at the NMJ, amplified by calcium release from intracellular stores (1, 3, 5–10). These changes lead to weakness and impaired neuromuscular transmission through an effect on both pre- and postsynaptic determinants of synaptic transmission, including quantal release, synaptic cleft anatomy, and the function and density of postsynaptic AChRs. Expression of several distinct AChR mutations characteristic of the SCS in transgenic mice reproduces the features of the SCS. The SCS mutation, εL269F, expressed in transgenic mice, most closely reproduces the clinical syndrome of weakness, fatigability, and impaired neuromuscular transmission (9, 10).

Elucidating the biochemical pathways coupling the ionic disturbance in SCS to the synaptic degeneration may be of benefit to understanding other degenerative disorders of nerve and muscle. Activation of endogenous proteolytic enzymes plays a prominent role in cell death, in muscular dystrophy, and in neurodegenerative diseases (11–13). We previously showed that some forms of the cysteine proteases, caspases that play a prominent role in the apoptotic cascade, are locally activated at the NMJ in SCS and in its transgenic mouse model (14, 15). Calcium overload of muscle fibers that is highly localized to the NMJ in SCS might exhaust calcium buffering reserves and activate the calcium-activated cysteine protease calpain. Previous attempts to detect activated calpain polypeptide in Western blots in SCS muscle homogenates were unsuccessful, perhaps due to the localized nature of the process. In this study we employed the enzymatic substrate, t-butoxycarbonyl-Leu-Met-7-amino-4-chloromethyl-coumarin (BOC-Leu-Met-CMAC), a fluorogenic calpain substrate used to detect calpain-like activity within living cells. The nonfluorescent BOC-Leu-Met-CMAC molecule is membrane permeable and becomes nondiffusible by enzymatic conjugation to thiol groups by glutathione-S-transferase (16). Proteolytic cleavage at the methionine residue releases and unquenches the fluorescent membrane-insoluble MAC-thiol moiety.

We investigated the role of calpain in impaired synaptic transmission seen in SCS. We found that calpain activity in muscle fibers from εL269F SCS transgenic mice was significantly elevated at the NMJ, which was dependent on synaptic activity, and diminished
to baseline with AChR blockade. Transgenic expression of human calpastatin (CS), the natural inhibitor of calpain, in εL269F mice reduced calpain to baseline, normalized NMJ size and miniature endplate current (MEPC) frequency and improved strength and neuromuscular transmission. Persistent endplate myopathy in CS-SCS double transgenic mice was associated with ongoing, localized activation of components of the caspase family of cysteine proteases, which are not inhibited by CS. These findings provide evidence for the role of 2 separate families of cysteine proteases in the SCS, for which separate therapeutic strategies for inhibition may provide synergistic benefit.

Results

Muscle calpain activity is elevated in a transgenic model of SCS. Using dissociated muscle fibers from the flexor digitorum brevis (FDB) loaded with the fluorogenic calpain substrate BOC-Leu-Met-CMAC, we compared the fluorescent signal from SCS and control fibers taken from mice aged 4–8 months. Many FDB fibers from εL269F mice had visibly elevated fluorescence, some with regions suggesting intense calpain activity localized to endplate areas (Figure 1A), while nontransgenic control fibers (WT) had less intense fluorescence (Figure 1B). The mean fluorescence signal intensity of εL269F SCS fibers at endplates was approximately 2-fold greater than that of WT fibers (Figure 1C; 1,591 ± 72 vs. 815 ± 72; n = 10; P < 0.01). We confirmed the specificity of these signals by preincubation with vehicle or with a specific peptide inhibitor of calpain, calpain inhibitor 1 (CP1). CP1 treatment of εL269F fibers reduced calpain to baseline, normalized NMJ size and miniature endplate Ca\textsuperscript{2+} after axotomy, we tested whether calpain activity in SCS muscle was similarly dependent on muscle innervation. Surgical axotomy significantly reduced calpain activity of εL269F FDB fibers (Figure 2B; 761 ± 78) by 24 hours but had no effect on the contralateral, innervated FDB fibers (1,447 ± 73; n = 3; P < 0.01). To insure that axotomy did not activate calpain through cell-signaling events caused by nerve retraction or trophic factor withdrawal from muscle fibers, we tested the effect of irreversible blockade of AChR activation using α-bungarotoxin (αBT) (18). In preliminary experiments we established the dose of αBT needed to completely block AChRs in the FDB muscle. As with axotomy, complete blockade of AChRs in vivo caused a significant reduction in calpain activity assessed after 24 hours when compared with the untreated contralateral hind limb (Figure 2C; 1,009 ± 84 vs. 1,600 ± 98; n = 4; P < 0.01). Therefore, increased calpain activation in SCS requires continuous activation of mutant AChRs.

A CS transgene normalizes calpain activity in double-transgenic εL269F muscle. We hypothesized that increased calpain activity in SCS may contribute to the pathogenesis of the SCS. To test the protective effect of CS in SCS, we bred εL269F mice with mice bearing a human CS transgene targeted to skeletal muscle (CS mice) to generate mice bearing both the SCS and CS transgenes (CS/εL269F mice). Transgenic overexpression of CS in mdx mice improved dystrophic changes (19). We found that, as with incubation with CP1, CS reduced the level of calpain activation in εL269F mice.

Figure 1
Calpain activity is elevated in transgenic SCS muscle fibers. (A and B) Representative images of acutely dissociated εL269F transgenic (A) and WT (B) FDB muscle fibers after loading with 10 μM BOC-Leu-Met-CMAC and labeling endplates (white arrowheads) with αBT (pseudo-colored pink). (C) Average fluorescence intensity for εL269F transgenic or WT BOC-Leu-Met-CMAC–loaded muscle fibers after no treatment (Un) or treatment with vehicle (Vh) or peptide calpain inhibitor (CP1). Calpain activity of εL269F transgenic muscle fibers was significantly elevated when compared with that of WT (1,591 ± 72 vs. 815 ± 72; n = 10; P < 0.01). No substantial effect was observed for either genotype when the fibers were treated with vehicle (εL269F: 1,591 ± 72, n = 10, vs. 1,593 ± 72, n = 3, and WT: 815.1 ± 72, n = 10, vs. 855.3 ± 52, n = 3; P > 0.05). CP1 pretreatment of εL269F muscle fibers significantly reduced calpain activity (vehicle: 1,591 ± 72, n = 10, vs. CP1: 801.6 ± 25, n = 3; P < 0.01), with values similar to those obtained for WT animals (P > 0.05). Calpain activity of CP1-treated WT fibers was similar to that of untreated WT fibers (924 ± 34, n = 3, vs. 815.1 ± 72, n = 10; P > 0.05). Scale bar: 100 μm.
to baseline levels. Figure 3 compares the BOC-Leu-Met-CMAC fluorescence signal from muscle fibers of εL269F (Figure 3A), CS/εL269F (Figure 3B), control CS (Figure 3C), and WT mice (Figure 3D). Analysis of fluorescence intensity at muscle fiber end-plate regions (Figure 3E) revealed that the increased calpain activity in εL269F muscle fibers was significantly reduced by the presence of the CS transgene (1,607 ± 79, n = 9, vs. 990.0 ± 145; n = 5; P < 0.05) and that calpain activity of CS (1,029 ± 83; n = 5) and control (WT, 819.0 ± 92; n = 8) was similar in the populations of fibers (P = 0.83 and P = 0.34, respectively) when compared with CS/εL269F. Finally, to validate the specificity and consequences of the measured changes in calpain activity with respect to known calpain substrates, we used immunoblotting with antibodies for specific calpain substrates. Figure 3F shows results of an immunoblot for troponin-I, a calpain substrate, using muscle homogenates that suggests that the 24-kDa troponin-specific bands induced by quantal release of ACh. It has an exponential basis for the improved neuromuscular transmission caused by the expression of CS corrects MEPC frequencies. Impaired synaptic function in SCS is due to a combination of effects of the mutant AChR on the pre- and postsynaptic determinants of successful neuromuscular transmission. To investigate the electrophysiological basis for the improved neuromuscular transmission caused by inhibition of calpain in SCS mice, we evaluated synaptic function by 2-electrode voltage-clamp analysis. This in vitro technique allows recording of spontaneous MEPCs in excised diaphragm muscle. Figure 6, A–D, shows representative recordings of MEPCs from εL269F mice (Figure 6A), CS/εL269F mice (Figure 6B), CS mice bearing the CS transgene alone (Figure 6C, CS), and nontransgenic littermates (Figure 6D, WT). MEPC recordings from each genotype were analyzed with respect to 3 parameters: MEPC decay rate (Figure 6E), MEPC amplitude (Figure 6F), and MEPC frequency (Figure 6G).

The MEPC is the reflection of the opening of the AChR channels induced by quantal release of ACh. It has an exponential decay phase whose rate can be defined by the time constant (τ) of this exponential decay function, which, in turn is proportional

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MEPC decay phase analysis is depicted in Figure 6E. The decay (increased MEPC in neuromuscular transmission in SCS, prolonged decay phases that slow the closure of the channel are a hallmark of this disease.

P for control mice (\(8.29 \pm 0.75; n = 26\), vs. \(1.607 \pm 0.79\), n = 9; \(P < 0.05\)). This reduction was similar to levels observed in CS (1,029 ± 83; n = 5) and WT mice (819.0 ± 92; n = 8; \(P > 0.05\)). No significant difference was observed when CS/L269F, CS, and WT mice were compared (\(P > 0.05\)). (F) Western blot of FDB muscle homogenates probed with troponin-I (Trop-I; 24 kDa) antisera with loading control (IgG heavy chain, 55 kDa), illustrating protection of full-length troponin I in CS/L269F mice. (G) Upper panel, CS: muscle homogenates (2 mice for each genotype) probed with monoclonal anti-CS antibody demonstrating that the strong signal for transgenic human CS compared with mouse CS is not affected by coexpression of the L269F transgene. Lower panel, LC: Ponceau red staining showing actin band for loading control. Scale bar: 100 μm.

Figure 3
Transgenic CS overexpression normalizes calpain activity in double-transgenic L269F FDB fibers. (A–D) Representative images of FDB muscle fibers from L269F (A), CS/L269F (B), CS (C), and nontransgenic, WT mice (D). (A) L269F muscle fibers occasionally revealed intense regions of calpain activity near or colocalized to the NMJ (inset, ×4 to original). (E) Calpain activity was significantly reduced in CS/L269F double-transgenic mice compared with L269F mice (990.0 ± 145, n = 5, vs. 1,607 ± 79, n = 9; \(P < 0.05\)). This reduction was similar to levels observed in CS (1,029 ± 83; n = 5) and WT mice (819.0 ± 92; n = 8; \(P > 0.05\)). No significant difference was observed when CS/L269F, CS, and WT mice were compared (\(P > 0.05\)). (F) Western blot of FDB muscle homogenates probed with troponin-I (Trop-I; 24 kDa) antisera with loading control (IgG heavy chain, 55 kDa), illustrating protection of full-length troponin I in CS/L269F mice. (G) Upper panel, CS: muscle homogenates (2 mice for each genotype) probed with monoclonal anti-CS antibody demonstrating that the strong signal for transgenic human CS compared with mouse CS is not affected by coexpression of the L269F transgene. Lower panel, LC: Ponceau red staining showing actin band for loading control. Scale bar: 100 μm.

The frequency of spontaneous quantal release of MEPCs in L269F mice was severely diminished when compared with that in nontransgenic littermates (Figure 6F; 0.29 ± 0.02 s\(^{-1}\), n = 23, vs. 0.57 ± 0.02 s\(^{-1}\), n = 57; \(P < 0.05\)). This presynaptic change, also seen in some SCS patients (1, 2), can be attributed to the severe remodeling and disruption in endplate architecture in SCS and likely contributes significantly to impaired neuromuscular transmission through reduced quantal content and reduced amplitude of the evoked endplate potentials. In CS/L269F mouse diaphragm, the MEPC frequency was increased by approximately 2-fold compared with that in L269F mice (Figure 6G; 0.51 ± 0.06 s\(^{-1}\), n = 22, vs. 0.57 ± 0.02 s\(^{-1}\), n = 23) and similar to that in normal mice. MEPC frequency was also approximately doubled in CS mice when compared with that in nontransgenic mice (1.17 ± 0.17 s\(^{-1}\), n = 14, vs. 0.57 ± 0.02 s\(^{-1}\), n = 57 NMJs; \(P < 0.05\)). Increased MEPC frequency in muscle from mice overexpressing a calpain inhibitor in muscle is consistent with the demonstrated role of calpain and other proteases in synaptic withdrawal, based on the effect of calpain inhibitors in stabilizing synaptic contacts (22–25).

Molecular and structural correlates for improved strength and neuromuscular transmission in SCS. Reduced MEPC amplitudes in SCS and other NMJ disorders are attributed, in part, to a reduced number of AChR subunits at the NMJ. We previously demonstrated that endplate AChRs are reduced in CS/L269F mouse diaphragm, the MEPC frequency was increased by approximately 2-fold compared with that in L269F mice (Figure 6G; 0.51 ± 0.06 s\(^{-1}\), n = 22, vs. 0.57 ± 0.02 s\(^{-1}\), n = 23) and similar to that in normal mice. MEPC frequency was also approximately doubled in CS mice when compared with that in nontransgenic mice (1.17 ± 0.17 s\(^{-1}\), n = 14, vs. 0.57 ± 0.02 s\(^{-1}\), n = 57 NMJs; \(P < 0.05\)). Increased MEPC frequency in muscle from mice overexpressing a calpain inhibitor in muscle is consistent with the demonstrated role of calpain and other proteases in synaptic withdrawal, based on the effect of calpain inhibitors in stabilizing synaptic contacts (22–25).

As shown previously, MEPC amplitudes of L269F mice were diminished when compared with those of nontransgenic littermates (Figure 6F; 1.61 ± 0.12 nA, n = 26, vs. 2.20 ± 0.10 nA, n = 31; \(P < 0.05\)), a change attributed to the combined effects of the disrupted synaptic architecture and the reduced number of endplate AChRs (1, 9). MEPC amplitudes (Figure 6F; 1.39 ± 0.03 nA, n = 27, vs. 1.61 ± 0.12 nA, n = 26; \(P = 0.04\)) were slightly smaller in CS/L269F mice compared with L269F mice, although the difference did not reach significance. However, the trend toward reduced MEPC amplitudes associated with the CS transgene contrasts with the improved synaptic function in CS/L269F mice and implies that a presynaptic, positive effect on synaptic function prevails.
inhibition acts presynaptically to increase synaptic contacts.

We investigated the structural basis for the improved MEPC frequency, which had a greater impact on synaptic function than did the slight change in MEPC amplitudes. Using calpain inhibitors in normal, suggesting that, as noted in other studies (22–25), calpain was not fully corrected to normal. These morphological findings were consistent with the action of calpain inhibitors in reducing endplate remodeling and stabilizing neuromuscular contacts.

To investigate the cause of the persistent endplate myopathy, we encoded the identity of the electron micrographs and scored them anonymously for presence or absence of this pattern of focal myofibrillar degeneration (Figure 8D). We found that 86% ± 7% of εL269F endplates had focal myofibrillar abnormalities, compared with only 30% ± 16% of CS/εL269F endplates (P < 0.05; n = 3 mice). Myofibrillar abnormalities in CS/εL269F endplates were more prevalent than in controls, but were not statistically different from either nontransgenic (4.7% ± 2.3%; n = 3; P = 0.32) and CS transgenic littersmates (7.0% ± 4.1%; n = 4; P = 0.35). Moreover, there was no statistical difference in myofibrillar abnormalities identified in CS-overexpressing mice compared with nontransgenic littersmates. Thus, improvement of strength in CS/εL269F mice may result, in addition, from factors distal to AChR activation, such as an effect on muscle fiber contractile force.

CS does not prevent caspase activation at εL269F NMJs. Finally, to investigate the cause of the persistent endplate myopathy, we

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**Figure 4**

CS overexpression reduces weakness in εL269F mice. CS/εL269F mice performed significantly better than did age-matched εL269F mice on the wire-hang test (67 ± 8, n = 9, vs. 16 ± 5, n = 8; P < 0.05). No difference in strength scores was detected between CS and WT age-matched mice (96 ± 2, n = 4, vs. 98 ± 1, n = 5; P > 0.05).

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**Figure 5**

CS overexpression improves neuromuscular transmission in εL269F mice. (A–D) Representative recordings of CMAP recorded over the hind foot and evoked by repetitive stimulation of the sciatic nerve for mice of the genotypes εL269F (A), CS/εL269F (B), CS (C), and WT (D). (A) εL269F mice had decremental CMAP evoked by repetitive stimulation. (B) CS/εL269F mice had significantly smaller decremental response in the CMAP than observed in εL269F transgenic mice (A) (P < 0.05). Decrements in CS transgenic (C) and WT (D) were statistically indistinguishable (E; εL269F: 17 ± 3.2, n = 8, vs. CS/εL269F: 9.0 ± 1.3, n = 8, vs. CS: 3.6 ± 1.2, n = 6, vs. WT: 3.4 ± 0.75, n = 7 mice).
are present at the NMJ in muscle biopsies of SCS patients and in
tensively). Numerous associated FDB muscle fibers to compare caspase enzymatic activity
onstrated that activated forms of the cysteine protease caspases
indistinguishable. To confirm that enzymatic activity correlated
the cleaved form of caspase-3 and compared sections of brachioradialis muscle biopsies from eL269F and CS/eL269F double transgenic mice (Figure 9, C and D, respectively). As with muscle from patients, numerous muscle fibers were labeled in and around the endplate region with active caspase-3 antibody. The number of motor endplates labeled with active caspase-3 antibody was the same in eL269F mice and CS/eL269F double-transgenic mice (Figure 9E; 54% ± 5% vs. 51% ± 5%; n = 3; P = 0.59) and increased roughly 4-fold when compared with that in nontransgenic littermates (54% ± 5% vs. 12% ± 4%; n = 3 mice; P < 0.05). No significant difference was noted when comparing caspase-labeled endplates in CS-overexpressing and nontransgenic mice (14% ± 4% vs. 12% ± 4%, n = 3 mice, P = 0.76). Thus, inhibition of calpain or the presence of overexpressed CS protein in SCS mice does not alter the extent of caspase-3 activation.

**Discussion**

A clearer understanding of how disturbed AChR channel gating and leaky synaptic ion channels give rise to progressive impairment of synaptic function in SCS will enhance our ability to understand a variety of diseases in which synapse function is affected, including epilepsy and Alzheimer disease. In this study we have demonstrated that in intact, functioning synapses from mice expressing mutant AChRs from SCS, the calcium-activated protease calpain is activated and plays a role in promoting neuromuscular disease. The increased calpain activity is dependent on the continued activity of mutant AChRs and ceases after receptor blockade or axotomy, coincident with the disappearance of accumulated calcium. Coexpression of a CS transgene in the eL269F mice partially corrects strength and neuromuscular transmission, through a presynaptic protective effect on synaptic size and strength, and to a lesser extent focal myofibrillar degeneration. Features of the endplate myopathy, such as nuclear and mitochondrial degeneration, persist despite inhibition of calpain. These are associated with continued activation at the NMJ of a separate cascade of cysteine proteases whose activity is not affected by CS (26, 27). These findings suggest that CS improves neuromuscular transmission in eL269F mice predominantly by strengthening the synaptic contacts, rather than through correcting the postsynaptic side of the NMJ. Calpain inhibition may also provide some local protection of contractile apparatus. The calcium-activated neutral protease calpain is a cysteine protease of ubiquitous tissue distribution and broad subcellular localization (28–30). In muscle, calpain is immunologically localized to endomysial collagen fibrils, basal lamina, sarcolemma, Z bands, and NMJs, as well as the extracellular space (29, 31). Calpains exist in multiple subtypes, differing in calcium sensitivity and function, and are involved in several cellular signaling pathways, myoblast fusion, cytoskeletal protein turnover, neurite outgrowth, and growth cone turning in development (26, 32–35). In the neuromuscular system, during development and reinnervation, calpain and possibly other proteases play a prominent role in synaptic remodeling and stabilization of neuromuscular contacts, a process that is highly reminiscent of that observed in SCS (24, 25, 32, 36–39). Calpain activation may also contribute to the pathogenesis of stroke, myocardial infarction, Alzheimer disease, and muscle disorders such as muscular dystrophy. mdx mice, which bear mutations in the murine form of dystrophin, have a muscle disease similar to Duchenne muscular dystrophy (40). mdx mice bearing the same human CS transgene used in this study have less severe dystrophic pathology (19).
Elevated calpain activity in εL269F mice appears either as a diffuse increase or as intense regions of calpain activity located near the NMJ. The focal activity of calpain is consistent with previous findings of histochemically detectable deposits of calcium at NMJs in muscle biopsies from SCS patients or transgenic mice or localized increases detected in SCS muscle in real time using calcium-sensitive dyes in vitro (1, 9–11, 15, 16). The rapid disappearance of elevated calpain activity after axotomy or AChR blockade demonstrates that the increased calpain activity is dynamic and directly related to ongoing activity of mutant AChRs rather than to a compensatory response.

The improvement in strength and neuromuscular transmission seen in εL269F transgenic mice expressing CS appears to result predominantly from a strengthening of synaptic connections, leading to normalized endplate size and MEPC frequency, rather than a protective effect on AChRs. Rather than being increased, both MEPC amplitude and AChR number were normal or slightly increased ca.

ulation of nerve terminal retraction (see below) may protect compromised synaptic regions that would otherwise be eliminated and increase the pool of smaller-amplitude MEPCs.

The increase in synaptic strength in SCS mice bearing the CS transgene is consistent with the recognized participation of calcium-activated proteases in nerve terminal elimination of polyneuronal innervation, in stabilizing neuromuscular contacts, and in remodeling of the NMJ. These processes are modulated by protease inhibitors that appear to protect calpain targets in the muscle and nerve cytoskeleton and basal lamina (22–25). Given the prominent activity of calpain in mouse SCS muscle fibers, it is not surprising that this enzyme participates in the NMJ remodeling recognized in SCS. The current model expands on those of previous protease inhibitor studies, as it involves a chronic, degenerative neuromuscular disease and direct transgenic targeting of calpastatin expression to muscle. Nevertheless, the same functional targets appear to be protected. This is consistent with the observation in fusing myoblasts that muscle-derived calpain is exteriorized and acts on extracellular matrix components, such as fibronectin (33). Last, the ultrastructural and immunoblot studies also point to some protection by calpain inhibition of myofibrillar structural proteins, recognized targets of calpains, indicating the action and potential role of this enzyme in muscle disease (41–43).

Calpain inhibition by CS expression did not improve several significant features of the endplate myopathy in SCS mice, including degenerating nuclei and dilated mitochondria. A second family of cysteine proteases, the caspase proteases, makes up a cascade of enzymes that underlie apoptotic cell death mechanisms and are

Figure 8
CS overexpression partially corrects the endplate myopathy in εL269F mice. Representative electron micrographs comparing εL269F (A), CS/εL269F (B), and WT (C) ultrastructure at the NMJ. Many of the classical features of endplate myopathy were still present in εL269F and CS/εL269F mice, such as the degenerating subjunctional nuclei with condensed chromatin and vacuoles in these NMJs. However, myofibrils appeared to be more disorganized and blurred focally in the subjunctional area of NMJs from εL269F transgenic mice (A, arrowhead). (D) When evaluated, the myofibrillar blurring was significantly less \( P < 0.05 \) at CS/εL269F endplates compared with εL269F endplates (εL269F: 86% ± 7%, \( n = 3 \), vs. CS/εL269F: 30% ± 16%, \( n = 3 \), vs. CS: 7.0% ± 4.1%, \( n = 4 \), vs. WT: 4.7% ± 2.3%, \( n = 3 \) mice, 8–10 endplates/animal). Scale bars: 2.6 μm.

Figure 7
CS expression improves endplate size but not endplate myopathy. Fluorescence imaging of muscle fibers labeled with of RoB. The mean size of endplate regions in εL269F mice was smaller than that in control (WT) endplates (\( P < 0.05 \)). In contrast, endplate size in CS/εL269F mice was significantly increased 2-fold when compared with that in εL269F mice (\( P < 0.05 \)) but not quite corrected to that of CS or normal nontransgenic mice (εL269F: 33 ± 1.9 μm², \( n = 248 \), vs. CS/εL269F: 62 ± 5.4 μm², \( n = 139 \), vs. CS: 84 ± 3.4 μm², \( n = 237 \), vs. WT: 87 ± 4.8 μm², \( n = 148 \) endplates for 4 animals/genotype). Endplate size of mice expressing CS alone was not different from normal (\( P = 0.59 \)).
involved in a complex cross-talk with the calpains (44). Caspases are also activated at the NMJ in SCS, and organellar degeneration resolves in close parallel to the disappearance of caspase activity (15, 16). However, activated caspases persist at the NMJ in SCS after calpain inhibition by CS (Figure 9). Studies have shown that activated caspases also occur at the NMJ in SCS, and organellar degeneration resolves in close parallel to the disappearance of caspase activity in SCS and related excitotoxic and muscle disorders.

Methods
Animals and reagents. The generation, characterization, and screening of rL269F slow-channel transgenic mice (rL269F mice) and mice expressing human CS have been reported previously (9, 19, 46). rL269F transgenic mice, line 14, express high levels, approximately 1,000-fold over endogenous WT mouse α2 subunit, of the mouse AChR α2 subunit mRNA bearing an EL269F mutation under the control of the muscle creatine kinase promoter (MCK). CS-transgenic mice, line 70.7, were derived from a construct that expresses human CS under the control of the human β-actin promoter and expresses CS protein at a level about 30-fold greater than endogenous protein. All breeding, screening, and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Minnesota. Double-transgenic mice bearing both transgenes were detected in F1 litters by PCR analysis of tail DNA using transgene-specific primers and were viable. Control mice (WT) consisted of hybrid littermates or age-matched transgene-negative mice. Age-matched mice, approximately 2–8 months, were used for all studies.

RoBT and BOC-Leu-Met-CMACE (prepared as a 10-mM stock in DMSO) were obtained from Molecular Probes (Invitrogen). SR-VAD-FMK was acquired from BIOMOL International. N-acetyl-Leu-Leu-Ne-Leu-CHO (CP1) was purchased from Calbiochem. Dulbecco’s modified Eagle’s medium without phenol red (DMEM) and Hank’s solution without phenol red (Hanks) were from Invitrogen. Mouse monoclonal troponin-I (skeletal) antibody was from Abcam. Rabbit polyclonal anti-Bax and goat polyclonal troponin-I were purchased from Santa Cruz Biotechnology Inc. Secondary antibodies conjugated to HRP and ECL reagents were from Amersham. Anti-goat secondary antibody conjugated to HRP was purchased from Jackson Immunoresearch Laboratories Inc. Nitrocellulose and PVDF membranes were from Bio-Rad. Protease inhibitor cocktail Complete Mini tablets (EDTA-free) were from Roche. BCA protein assay reagents were from Pierce. All others were purchased from Sigma-Aldrich unless otherwise specified.

Animals were sedated as needed for surgery using Avertin (250 mg/kg i.p.) (47) and euthanized immediately after the surgery. Strength testing by wire-hang test, measurement of AChR density by [125I]βBT binding, and electromyography were performed as described previously (8, 9). Briefly, the wire-hang test was performed by timing the period the animals were able to hang suspended gripping a wire by their forepaws. AChR density was determined as the ratio of [125I]βBT binding on labeled muscle bundles to the number of endplates in the fibers. Electromyography was performed on anesthetized animals by recording CMAPs using subcutaneous electrodes placed over heel and at toe and evoked by direct stimulation of the sciatic nerve. Voltage-clamp analysis was performed in excised diaphragm in vitro using sharp electrodes inserted into individual muscle fibers, 1 for recording voltage and 1 for injecting current. The signals are amplified and digitized using a voltage-clamp amplifier and the software program pCLAMP (Molecular Devices). Histochemical (tissue) stains for acetylcholinesterase and GBHA were described previously (48, 49). Caspase-3 immunostaining and microscopy were performed as previously described (16). Voltage-clamp analyses of MEPCs were performed exactly as described previously (8).

Axotomy and AChR blockade. The left sciatic nerve was exposed through a 5-mm incision in the upper thigh in anesthetized animals, and a 2-mm nerve. The hind-limb FDB muscles were aseptically exposed and excised, and fibers were dissociated by incubation in 400 μl digestion solution (3% collagenase, 1% hyaluronidase, and 5% BSA in DMEM), at 37° C with 5% CO2 for 1.5 hours. To avoid muscle fiber damage and contraction, all fiber suspensions were allowed to settle by

Figure 9
CS does not protect rL269F NMJs from caspase activation. Liberated rL269F (A) and CS/rL269F FDB (B) muscle fibers were stained with SR-VAD-FMK (red) to detect active caspase-3 activity or Alexa Fluor 488–conjugated αBT (AF488–αBT; green) to localize the NMJ. Skeletal muscle sections from rL269F (C) and CS/rL269F (D) were fluorescently double-labeled with anti–active caspase-3 antibody (red) and AF488–αBT (green) to localize the NMJ. Note the colocalization of the fluorescent signals demarcated by the arrowheads. (E) When the frequency of colabeling was evaluated in a blinded manner, rL269F mice had a roughly 4-fold increase in frequency of activated caspase-3 at the NMJ when compared with CS and nontransgenic littermates (P < 0.05); no significant difference (P = 0.59) was found when comparing rL269F and CS/rL269F mice. Results for CS mice and nontransgenic littermates were statistically indistinguishable (P = 0.76) (rL269F: 54±5%, n = 4, vs. CS/rL269F: 50±5%, n = 4, vs. CS: 14±4%, n = 3; vs. WT: 12±4%, n = 3 mice). Scale bars: 10 μM (A and B), 5 μM (C and D).
gravity sedimentation in 0.7 ml PCR tubes for 20 minutes and the supernatants discarded. Controls consisted of contralateral dissociated FDB muscle fiber suspensions incubated in 400 μl 80-μM CP1 or 1% DMSO (vehicle) in DMEM at 37°C with 5% CO2 for 20 minutes.

Fiber bundles were transferred to a labeling cocktail consisting of 10 μM BOC-Leu-Met-CMAC, 0.3 μM RoBT, 1 μM ATP, and 5% horse serum in Hanks solution without phenol red (Invitrogen), and, after removal of tendinous material, triturated ×4 with a wide bore polyethylene plastic pipette. The fiber suspensions were washed once in 700 μl Hanks prior to mounting (in Hanks) on glass slides.

Staining with SR-VAD-FMK was performed after dissociation as described above and according to the manufacturer’s instructions, except that the fibers were washed extensively in a diffusion chamber overlaid with a micromesh with Hanks prior to visualizing.

Western immunoblotting. FDB whole muscle or muscle fiber preparations were disrupted in frosted glass homogenizers in 5 volumes of protein extraction buffer thoroughly while on ice (20 mM phosphate buffer, pH 7.8, 10 mM EDTA, 10 mM EGTA, plus 1 tablet of protease inhibitor cocktail). Crude protein concentration was estimated using the BCA protein assay, and samples were heat treated with standard loading buffer under reducing conditions; loaded equally at 25 μg/well across all lanes; and electrophoresed on 10%–20% Bio-Rad Ready Gels. Sister gels were transferred in Hanks solution without phenol red (Invitrogen), and, after removal of the 465-nm image emissions were collected for 70 min with a micromesh with Hanks prior to visualizing.

After blocking in 5% milk-TBS, primary antibody dilutions were prepared in 5% milk-TBS and were 1:500 for rabbit polyclonal anti-Bax (data not shown). Monoclonal anti-CDK5 antibody (Takara Biomedicals) was used at 1:1,000. Secondary antibodies were prepared in 1% milk-TBS; the dilutions were 1:1,500 (or 1:10,000) for anti-mouse and -rabbit and 1:10,000 for anti-goat. Membranes were activated with ECL Chemiluminescence Detection System (Pierce Biotech) or ChemiGlow (Alpha-InnoTech), according to the manufacturer’s instructions, and exposed to Kodak X-OMAT or Pierce CL-Xposure film. As a loading control, a sister gel of the same homogenates was used to detect the 55-kDa species corresponding to IgG heavy chain (Figure 3F, upper panel).

Statistics. For comparison of 2 groups, data were compared using 2-tailed Student’s t test when assuming unequal variances. For comparison of 3 or more groups, data were analyzed using ANOVA followed by Bonferroni or Dunn’s post-hoc analysis. Data are reported as mean ± SEM. Overall experimental significance was assigned for individual comparisons when P was less than 0.0083, with a 95% confidence level (overall experimental significance, P < 0.05). A contralateral comparison strategy was applied where applicable, so one could compare treated and untreated sides within the same animal to eliminate concerns of individual genetic variation.

Note added in proof: Chen et al. recently reported that calpain, activated by cholinergic stimulation and inhibited by rapsyn, drives CDK5 activity, which dispenses AChR clusters in developing muscle fibers (50). This finding identifies additional NMJ proteins that may be involved in SCS pathogenesis.

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