Activated calcineurin ameliorates contraction-induced injury to skeletal muscles of *mdx* dystrophic mice

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Utrophin expression is regulated by calcineurin and up-regulating utrophin can decrease the susceptibility of dystrophic skeletal muscle to contraction-induced injury. We overexpressed the constitutively active calcineurin-A α in skeletal muscle of *mdx* dystrophic mice (*mdx*CnA*) and examined the tibialis anterior muscle to determine whether the presence of activated calcineurin promotes resistance to muscle damage after lengthening contractions. Two stretches (10 s apart) of 40% strain relative to muscle fibre length were initiated from the plateau of a maximal isometric tetanic contraction. Muscle damage was assessed 1, 5 and 15 min later by the deficit in maximum isometric force and by quantifying the proportion of muscle fibres staining positive for intracytoplasmic albumin. The force deficit at all time points after the lengthening contractions was approximately 80% in *mdx* muscles and 30% in *mdx CnA muscles. The proportion of albumin-positive fibres was significantly less in control and injured muscles from *mdx CnA** mice than from *mdx* mice. Compared with *mdx* mice, mean fibre cross-sectional area was 50% less in muscles from *mdx CnA** mice. Furthermore, muscles from *mdx CnA** mice exhibited a higher proportion of fibres expressing the slow(er) myosin heavy chain (MyHC) I and IIa isoforms, prolonged contraction and relaxation times, lower absolute and normalized maximum forces, and a clear leftward shift of the frequency–force relationship with greater force production at lower stimulation frequencies. These are structural and functional markers of a slower muscle phenotype. Taken together, our findings show that muscles from *mdx CnA** mice have a smaller mean fibre cross-sectional area, a greater sarcolemmal to cytoplasmic volume ratio, and an increase in utrophin expression, promoting an attenuated susceptibility to contraction-induced injury. We conclude that increased calcineurin activity may confer functional benefits to dystrophic skeletal muscles.**

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Duchenne muscular dystrophy (DMD) is the result of a mutation in the dystrophin gene such that the functional protein is not expressed. Dystrophin is essential for maintaining muscle membrane integrity in quiescent and contracting muscle fibres (Petrof *et al.* 1993; Pasternak *et al.* 1995; Lynch *et al.* 2000). The absence of dystrophin results in the loss of all dystrophin-associated proteins and the disruption of the linkage between muscle fibre cytoskeleton and the extracellular matrix (Matsumura & Campbell, 1994). Lack of dystrophin expression can increase the susceptibility of dystrophic muscles to contraction-induced injury (Dellorusso *et al.* 2002). In boys with DMD, a greater susceptibility to muscle damage and impaired muscle regeneration result in the progressive loss of muscle mass and strength and the replacement of viable muscle fibres with fat and connective tissue (Zammit & Partridge, 2005).

Increased susceptibility to muscle damage can be assessed using a variety of markers including increased plasma creatine kinase levels (Griggs & Rennie, 1983), inflammatory cell infiltration (Kissel *et al.* 2001) and greater myofibrillar protein catabolism (McKeran *et al.* 1977). In muscles from *mdx* mice (the *mdx* mouse is a model for DMD), susceptibility to contraction-induced injury can be assessed directly. Dellorusso *et al.* (2002) showed that after two maximal lengthening contractions (LCs) *in situ*, the force deficit of tibialis anterior (TA) muscles from *mdx* mice is four- to seven-fold greater than of TA muscles from wild-type (non-dystrophic) mice. Following repeated LCs *in vitro*, the force deficit

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of the extensor digitorum longus (EDL) muscles from *mdx* mice is five-fold greater than from wild-type mice (Deconinck *et al.* 1997). The magnitude of the force deficit following the LC protocol is indicative of the severity of muscle damage sustained. After the contraction-induced injury protocol, the proportion of TA or EDL muscle fibres with sarcolemmal damage can by assessed by quantifying intrafibre Procion orange or Alizarin red staining, which others have shown to be significantly greater in muscles from *mdx* mice compared to wild-type mice (Deconinck *et al.* 1997; Dellorusso *et al.* 2002).

Currently, the efficacy of pharmacological interventions to treat patients with DMD is limited. There is interest in developing a treatment strategy which would improve the resistance of dystrophic muscle to contraction-induced injury (Zammit & Partridge, 2005). One such strategy focuses on the up-regulation of utrophin. Utrophin has 80% homology with dystrophin and is normally localized at the neuromuscular junction (Deconinck *et al.* 1997). Utrophin can functionally compensate for dystrophin. Overexpression of full-length or truncated utrophin in muscles from *mdx* mice improved their muscle morphology, sarcolemmal stability and mechanical resistance to forced LCs (Deconinck *et al.* 1997; Tinsley *et al.* 1998).

Given the efficacy of utrophin for ameliorating the pathology of dystrophic muscles, there is interest in elucidating the cellular signalling pathways that regulate utrophin expression. Utrophin A protein levels are greater in slow-twitch muscles than in fast-twitch muscles and its expression is mediated by the calcineurin/NFAT (nuclear factor of activated T Cells) signal transduction pathway (Chakkalakal *et al.* 2003). Transgenic mice that express a constitutively active form of calcineurin-Aα (*CnA**) have four-fold greater utrophin mRNA levels and increased utrophin protein expression at the sarcolemma compared with wild-type mice (Chakkalakal *et al.* 2003). When these mice were crossed with *mdx* mice, the *mdx CnA** F1 offspring were dystrophin deficient and exhibited the hallmarks of increased calcineurin phosphatase activity including increased nuclear localization of NFAT, a slower muscle phenotype and increased utrophin expression (Chakkalakal *et al.* 2004). In muscles from *mdx CnA** mice, greater utrophin expression was associated with greater expression of all members of the dystrophin-associated protein complex, including β-dystroglycan, syntrophin and neuronal nitric oxide synthase (Chakkalakal *et al.* 2004). Increased utrophin expression and restoration of the dystrophin-associated protein complex improved the dystrophic pathology. In muscles from *mdx CnA** mice, inflammatory cell infiltration was attenuated, sarcolemmal stability was improved, as indicated by reduced cytoplasmic albumin and Evans blue staining, and plasma creatine kinase levels and the proportion of centrally nucleated fibres were also decreased (Chakkalakal *et al.* 2004).

In this study, we demonstrate an improved resistance to contraction-induced injury in TA muscles from *mdx CnA** mice. We examined the force deficit following two maximal LCs *in situ* and assessed the proportion of albumin-positive fibres as indicators of susceptibility to contraction-induced injury. In addition, we characterized the isometric contractile properties of TA muscles from *mdx CnA** mice and assessed the effect of expressing activated calcineurin in skeletal muscle on maximal force producing capacity.

Methods

Transgenic mice

All experiments were approved by the Animal Experimentation Ethics Committee of the University of Melbourne. Female *mdx* mice were mated with male transgenic mice (MCK (muscle creatine kinase)-CnA*) expressing a constitutively active form of *CnA** (O'Keefe *et al.* 1992). The MCK-CnA* male mice were bred on a B6C3F1 background and the female *mdx* mice had a C57BL/10 ScSn background. The transgenic F1 male offspring were identified by PCR screening of genomic DNA extracted from tail tissue (Naya *et al.* 2000). TA muscle function and susceptibility to contraction-induced injury was assessed *in situ* in 9- to 10-month-old *mdx CnA** mice ($n = 9$) and *mdx* ($n = 10$) mice.

In situ muscle function and LC protocol

The *in situ* TA muscle preparation, the isometric contractile properties of the preparation, and the LC protocol have been previously described (Schertzer *et al.* 2006). The mice were anaesthetized with 100 mg kg^{-1} ketamine and 10 mg kg^{-1} xylazine so that they were unresponsive to tactile stimuli. The mice were carefully monitored throughout the experiment and additional doses of anaesthetic were administered as required depending upon whether there was a reflex response to a toe pinch. Briefly, the distal portion of the TA muscle and its tendon were dissected from anaesthetized mice and the tendon was tied with 4.0 braided surgical silk to the lever arm of a dual-mode servomotor/force transducer (305-LR, Aurora Scientific Inc., Ontario, Canada). The TA muscle was stimulated by directly applying supramaximal square-wave pulses (10 V, 300 ms duration) to the sciatic nerve using a hook electrode to produce a maximum isometric contraction (Lynch *et al.* 2001*a*; Gregorevic *et al.* 2002). All stimulation parameters and contractile responses were controlled and measured using custom-built applications of LabView software (National Instruments, Austin, TX, USA) driving a personal computer with

an onboard controller (PCI-MIO-16XE-10, National Instruments) interfaced with the transducer-servomotor control/feedback hardware (305-LR; Aurora Scientific Inc.) (Lynch *et al.* 2001*a*; Gregorevic *et al.* 2002). Optimal muscle length (L_o) was determined from micro-manipulations of muscle length and a series of isometric twitch contractions. Maximal isometric tetanic force (P_o) was determined from the plateau of the frequency–force relationship. The muscles were stimulated at 10, 30, 50, 100, 150, 200, 250 and 300 Hz. Optimal fibre length (L_f) was determined by multiplying L_o by the TA L_f/L_o ratio of 0.6 (Schertzer *et al.* 2006).

To assess the susceptibility of TA muscles to LC-induced injury, muscles from *mdx CnA** mice and *mdx* littermates were subjected to two LC 10 s apart as previously described (Dellorusso *et al.* 2002; Schertzer *et al.* 2006). The muscles were stimulated at the frequency required to elicit P_o (i.e. 150 Hz) for 100 ms and then stretched to 40% of L_f at a velocity of 2 L_f s^{-1}. Stimulation during the LC was 200 ms in duration, for a total stimulation time of 300 ms. The muscle was held unstimulated in the lengthened position for 100 ms and then returned to L_o at a velocity of 2 × L_f s^{-1}. The muscles were allowed to recover for 1, 5 and 15 min, at which times P_o was reassessed (by stimulating the muscle maximally, i.e. at 150 Hz). The relative force deficit (loss of maximal force producing capacity) at 1, 5 and 15 min after the LCs was expressed as a percentage of the maximum P_o measured before the LCs. Overall muscle cross-sectional area was calculated by dividing muscle mass by the product of L_f and the density of mammalian skeletal muscle (1.06 mg mm^{-3}). Force per cross-sectional area, specific force (sP_o, in kN m^{-2}), was determined by dividing P_o by TA muscle cross-sectional area (Lynch *et al.* 2001*b*). At the end of the experiment, the still deeply anaesthetized mouse was killed by opening the thoracic cavity and surgically excising the heart.

Histology and immunohistochemistry

Sections (5 μm thick) of each muscle were cryosectioned and stained with haematoxylin and eosin (H&E). Images of cross-sections were captured with a digital camera (Spot, v2.2, Diagnostic Instruments, Sterling Heights, MI, USA) mounted on an upright microscope (BX-51, Olympus, Tokyo, Japan), and fibre circumference and myofibre number were determined using an image analysis system (AIS, v6.0, Imaging Research Inc., St Catherines, Ontario, Canada). The proportion of centrally nucleated fibres and mean fibre cross-sectional area were calculated by analysing ∼150 fibres per section. To assess variability in fibre size, the coefficient of variation (CV) for mean fibre cross-sectional area was calculated for each muscle analysed. To determine whether hyperplasia was a consequence of calcineurin activation, the number of myofibres was counted and divided by the area of muscle section examined. Approximately one-third of each muscle section was examined to quantify myofibre number.

For immunohistochemistry, sections were fixed in cold acetone for 15 min and endogenous peroxidase activity was inhibited with 1.5% H_2O_2 in 0.5 M Tris-buffered saline (TBS) for 5 min. Sections were incubated with blocking solution containing (%) normal goat serum 2, normal mouse serum 2, bovine serum albumin 1, cold fish gelatin 0.1, Triton X-100 0.1, Tween 20 0.05 and sodium azide 0.05, in 0.01 M phosphate-buffered saline (PBS; pH 7.2) for 30 min. A primary polyclonal antibody specific for utrophin A (sc-15377, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), diluted 1 : 60, a polyclonal antibody specific for albumin (Dako Corporation, Botany, Australia), diluted 1 : 4000, a monoclonal antibody specific for myosin heavy chain (MyHC) type IIa (Hybridoma Bank), diluted 1 : 40 or a monoclonal antibody specific for MyHC I (Hybridoma Bank), diluted 1 : 40, was added for 1 h. A secondary antibody, goat anti-rabbit secondary peroxidase-conjugated antibody (Dako Corporation) or biotinylated rabbit anti-mouse antibody (Dako Corporation) was added for 30 min. Visualization was preformed using a peroxidase detection system (Vector Laboratories) and the 3-amino-9-ethylcarbazole (AEC) + substrate chromogen (Dako Corporation).

To determine utrophin A content, three images of myofibres at 400 × magnification were captured for each uninjured TA muscle section exposed to the anti-utrophin A antibody using a digital imaging system (AIS, v6.0, Imaging Research Inc.). The percentage of the section that reacted with the utrophin antibody (and stained red with AEC) was quantified, averaged for each muscle, and expressed as percentage utrophin staining. To assess the proportion of muscle fibres positive for MyHC I and IIa, the number of muscle fibres that reacted with the respective antibodies were counted and expressed as a proportion of the total number of myofibres; ∼1000 fibres were examined for each muscle. To quantify muscle membrane damage, the number of myofibres that reacted with anti-albumin antibody were counted and expressed as a proportion of the total number of muscle fibres; ∼1600 fibres were examined for each muscle. The cross-sectional area of all albumin-positive fibres was also assessed.

Statistics

Values presented in all figures and tables are expressed as means ± S.E.M. All statistical analyses were performed using a statistics software package (Minitab Inc., State College, PA, USA). Two sample unpaired *t* tests were used to analyse differences in function and morphology in TA muscles from *mdx CnA** and *mdx* mice. Two-way ANOVA was used to assess differences in albumin staining

Table 1. Contractile properties of TA muscles from *mdx* and *mdx CnA mice**

		mdx	*mdx CnA**
	Body mass (BM; g)	38.8 ± 0.6	35.2 ± 0.7*
	Muscle mass (MM; g)	107.3 ± 2.8	92.6 ± 4.3*
	MM:BM	2.76 ± 0.07	2.34 ± 0.32
	Muscle CSA (mm^2)	11.8 ± 0.4	10.6 ± 0.6 ($P = 0.11$)
Twitch	P_t (mN)	681.8 ± 25.0	721.4 ± 30.7
	TPT (ms)	14.3 ± 0.5	19.0 ± 0.4*
	$^1/_2$RT (ms)	10.6 ± 0.6	19.3 ± 0.8*
	dP_t/dt (mN ms^{-1})	97.8 ± 3.0	84.5 ± 3.7*
Tetanus	P_o (mN)	2394.8 ± 52.5	1949.5 ± 84.3*
	sP_o (kN m^{-2})	204.5 ± 4.7	179.6 ± 8.3*

Values shown are means ± S.E.M. TA, tibialis anterior muscle; P_t, twitch force; TPT, time to peak twitch force; $^1/_2$RT, half relaxation time of twitch; dP_t/dt, rate of twitch force development; P_o, absolute maximum isometric force; sP_o, specific force (P_o normalized for muscle cross-sectional area). *Significant differences between *mdx* and *mdx CnA** mice are as indicated ($P < 0.05$).

Table 2. Analysis of fibre cross-sectional area in injured and uninjured TA muscles of *mdx* and *mdx CnA mice**

		Mean cross-sectional area (μm^2)			
		All fibres	Fibres with CN	Fibres without CN	Fibres positive for Albumin
mdx	Control TA	1988.3 ± 109.1 CV 74.6% ($n = 1779$)	1993.7 ± 116.6 ($n = 1201$)	1976.6 ± 98.9 ($n = 613$)	2383.3 ± 137.9 ($n = 526$)
	Injured TA	N/A	N/A	N/A	3080.6 ± 137.6 ($n = 856$)
*mdx CnA**	Control TA	1021.9 ± 57.6 CV 69.7% ($n = 1479$)	1032.4 ± 62.4 ($n = 865$)	991.4 ± 52.1 ($n = 603$)	1541.0 ± 124.5 ($n = 399$)
	Injured TA	N/A	N/A	N/A	1830.1 ± 240.1 ($n = 405$)

Values shown are means ± S.E.M. TA, tibialis anterior muscle; CN, central nuclei; CV, coefficient of variation; N/A, no analysis completed. CV is reported to illustrate the greater variability in fibre size in TA muscles from *mdx* and *mdx CnA** mice. Differences between specific groups are discussed in the Results.

between control and injured TA muscles from *mdx CnA** and *mdx* mice and to determine differences in the frequency–force curves for TA muscles from *mdx CnA** and *mdx* mice. Tukey's *post hoc* test was used to locate pair-wise significant differences, where appropriate. For all comparisons, $P < 0.05$ was considered significant.

Results

Histological analysis of dystrophic skeletal muscles overexpressing activated calcineurin

We generated *mdx CnA** mice to examine the effect of activation of the calcineurin signalling pathway in dystrophic muscle. Measurements of body mass and TA muscle mass showed lower values in *mdx CnA** mice than in *mdx* mice, but the ratio of muscle mass to body mass was similar (Table 1). Morphological studies of the muscles showed that the mean fibre cross-sectional area was two-fold greater in TA muscles from *mdx* than *mdx CnA** mice (Table 2 and Fig. 1). Muscle mass and whole muscle cross-sectional area were only ∼15% and 5% smaller, respectively, in muscles from *mdx CnA** compared to values in muscles from *mdx* mice, but the mean fibre size was ∼50% less in muscles from *mdx CnA**, suggesting an increase in the number of muscle fibres in TA muscles of *mdx CnA** mice. This is supported by our data indicating that the number of muscle fibres per mm^2 tissue was almost two-fold higher in TA muscles from *mdx CnA** than from *mdx* mice (Fig. 1).

To assess variability in fibre size, the CV for mean fibre cross-sectional area was calculated for each muscle analysed. The average CV for muscles from *mdx CnA** and *mdx* mice was ∼70%–75% (Table 2). Thus, overexpression of the constitutively active calcineurin transgene did not reduce variability in muscle fibre size.

In healthy muscle, nuclei are located at the periphery of the myofibre and the presence of central nuclei is an indicator of recently regenerated fibres, which is characteristic of myopathies where muscle fibres are more susceptible to on-going bouts of injury and repair. The proportion of myofibres with central nuclei was ∼10% lower in TA muscles from *mdx CnA** compared to muscles from *mdx* mice (Fig. 1). In both *mdx CnA** and *mdx* mice, the mean fibre cross-sectional area was similar for muscle fibres that were centrally nucleated and those that were not (Table 2).

To assess the muscle fibre-type proportions, histological sections were immunoreacted with antibodies recognizing MyHC I and IIa protein. We observed in TA muscles of *mdx* mice that 10% of myofibres expressed MyHC IIa protein and 3% expressed MyHC I protein, whereas 46% of the myofibres expressed MyHC IIa protein and 12% expressed MyHC I protein in TA muscles of *mdx CnA** mice (Fig. 2*A*). Thus, overexpression of activated calcineurin resulted in a four-fold increase in the number of type I and IIa fibres in dystrophic muscle.

Endogenous utrophin A expression was measured in TA muscles of *mdx* and *mdx CnA** mice (Fig. 2*B*). We observed that utrophin A protein expression was four-fold greater in TA muscles of *mdx CnA** than in muscles of *mdx* mice (Fig. 2*B*). Also, we noted a greater level of sarcolemmal utrophin staining (Fig. 2*B*) in muscle fibres from *mdx CnA** mice.

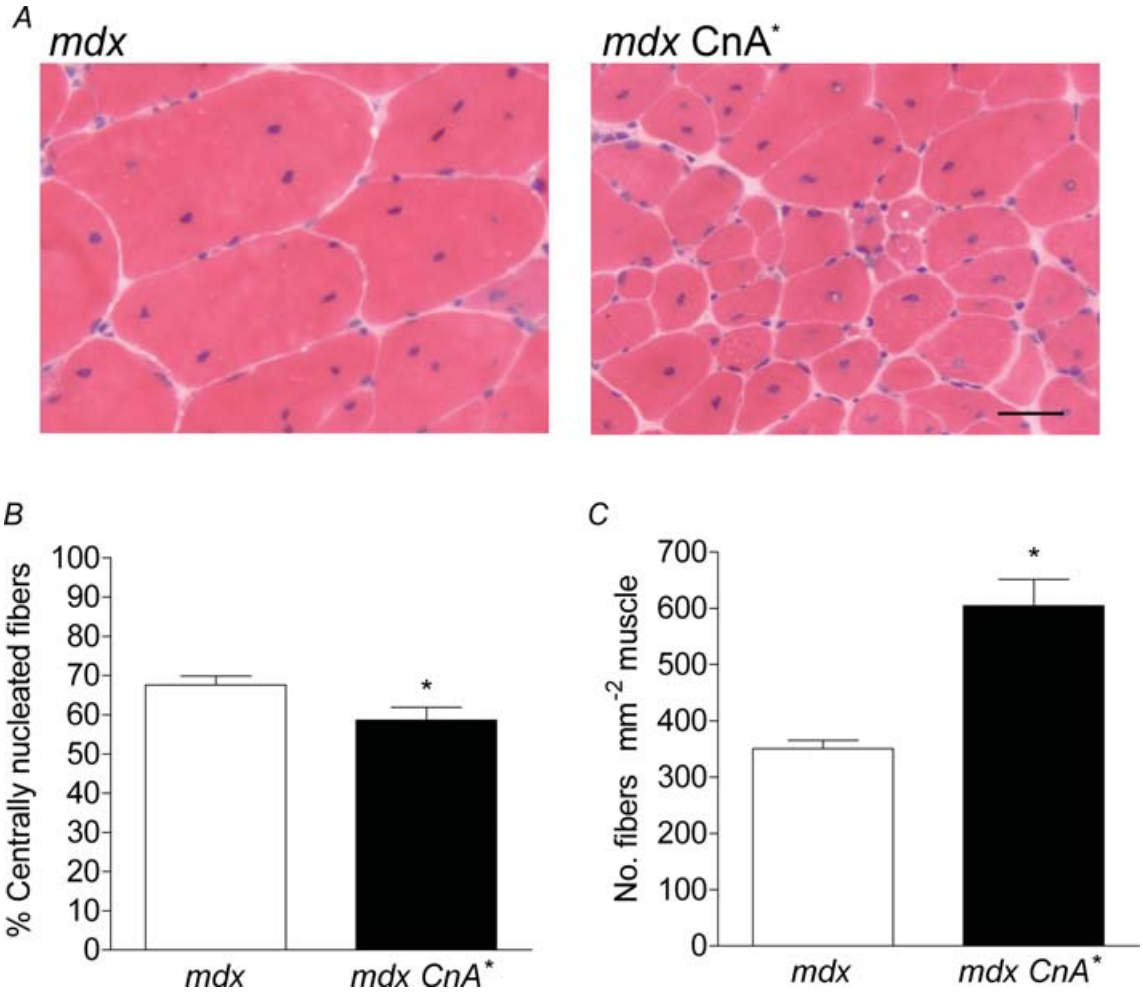


Figure 1. Proportion of centrally nucleated fibres and myofibre number in TA muscles of *mdx* and *mdx CnA mice**

A, representative H&E sections of uninjured TA muscles of *mdx* and *mdx CnA** mice. Note the variability in fibre size in muscles of *mdx* and *mdx CnA** mice. Scale bar represents 100 μm and applies to all panels. *B*, the proportion of centrally nucleated fibres was quantified to determine which muscle fibres had recently undergone regeneration. *Significant difference between TA muscles of *mdx* and *mdx CnA** mice ($P < 0.05$). *C*, the number of myofibres per mm^2 tissue was quantified to assess whether expression of the *CnA** transgene activated hyperplasia in TA muscles of *mdx* mice. *Significant difference between TA muscles of *mdx* and *mdx CnA** mice ($P < 0.05$).

Functional analysis of dystrophic skeletal muscles overexpressing activated calcineurin

When TA muscle function was assessed, a similar twitch force (P_t) was observed in muscles from *mdx CnA** and *mdx* mice; however, P_o was ∼20% greater in muscles from *mdx* mice. When P_o was normalized for muscle cross-sectional area, sP_o of muscles from *mdx CnA** mice was still ∼10% less than that of *mdx* mice (Table 1). The decrease in P_o and sP_o observed in TA muscles from *mdx CnA** mice compared with values in *mdx* mice, can be attributed to a greater proportion of Type IIa and type I fibres and a decrease in mean fibre cross-sectional area and muscle mass.

Characteristic of a slower muscle phenotype and the increase in type I and IIa fibres, we observed that the time-to-peak tension (TPT) and $^1/_2$ relaxation time ($^1/_2$RT) of muscles from *mdx* mice expressing the *CnA** transgene were prolonged compared to values in muscles from their control littermates (Table 1). Furthermore, at lower frequencies, 30 and 50 Hz, muscles from *mdx CnA** mice produced greater force than those from *mdx* mice; but at higher frequencies, 150 and 200 Hz, their force-producing capacity was lower (Fig. 3*A*). TA muscles of *mdx CnA** mice produced higher forces at the lower stimulation frequencies, as a result of the prolongation of $^1/_2$RT (Fig. 3*A*).

Activation of calcineurin reduces contraction-mediated injury in dystrophic muscles

After the first LCs (LC1), P_o was greater in muscles from *mdx* than *mdx CnA** mice. However, after the second LCs (LC2), TA muscles from *mdx CnA** mice produced more force (Fig. 3*B*). At all time points during recovery after LC1 and LC2, P_o was greater in muscles from *mdx CnA** than from *mdx* mice (Fig. 3*B*) and the relative force deficit was less (Fig. 3*C*). Given that the magnitude of the force deficit is related to the severity of muscle damage sustained, these findings show that muscles from *mdx CnA** mice are more resistant to contraction-induced injury than muscles from *mdx* mice.

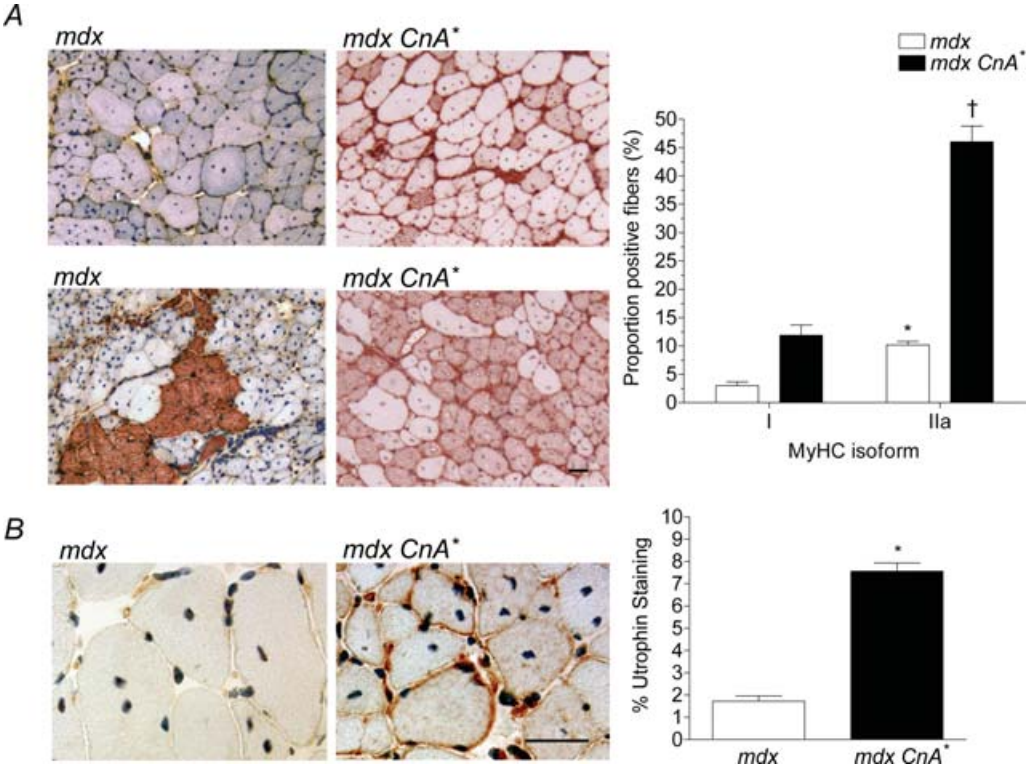


Figure 2. Calcineurin activation increased protein expression of the MyHC IIa isoform and utrophin A
A, data and representative images showing MyHC IIa-positive nuclei in TA sections of *mdx* and *mdx CnA** mice. Fibres expressing the MyHC IIa isoform stained red; ⊕ = positive. *Significant difference in the proportion of MyHC IIa fibres between TA muscles of *mdx* and *mdx CnA** mice ($P < 0.05$). Scale bar represents 100 μm and applies to upper panels. *B*, data and representative images showing utrophin A staining in TA sections from *mdx* and *mdx CnA** mice. Note the greater level of sarcolemmal utrophin A staining, seen as a red outline, in muscle fibres from *mdx CnA** mice. *Significant difference in utrophin A expression between TA muscles of *mdx* and *mdx CnA** mice ($P < 0.05$). Scale bar represents 100 μm and applies to lower panels.

Another marker of muscle damage is the proportion of albumin-positive fibres located in muscle. Following the two LCs, we observed a greater proportion of albumin-positive fibres in TA muscles from *mdx* than from *mdx CnA** mice (Fig. 4). In uninjured muscles from *mdx* mice, 4% of the myofibres stained positive for albumin, whereas this value was 7% in injured TA muscles. By comparison, 2% of fibres stained positive for albumin in uninjured muscles isolated from *mdx CnA** mice, and only 3% were positive for albumin in injured muscles from *mdx CnA** mice. These findings show that overexpression of activated calcineurin in muscles of *mdx* mice results in 50% fewer muscle fibres sustaining muscle membrane damage than in those muscles without activated calcineurin both in the basal state and after LCs.

We observed differences in the cross-sectional area of damaged myofibres isolated from *mdx* and *mdx CnA** mice. In muscles from *mdx* mice, the cross-sectional area of albumin-positive muscle fibres was greater than in muscle from the *mdx CnA** mice (Table 2). Furthermore, the cross-sectional area of albumin-positive fibres from injured TA muscles was greater than the cross-sectional area of albumin-positive fibres from uninjured TA muscles and also greater than the mean fibre cross-sectional area (Table 2). In *mdx* mice, the cross-sectional area of albumin-positive fibres from injured muscles was 55% greater than the mean fibre cross-sectional area and in *mdx CnA** mice it was 80% greater than the mean fibre cross-sectional area. In contrast, the cross-sectional area of albumin-positive muscle fibres from uninjured TA muscles of *mdx* mice was 20% greater than the mean fibre cross-sectional area and in *mdx CnA** mice it was 50% greater than the mean fibre cross-sectional area. These findings indicate that the smaller fibre cross-sectional area may mediate a protective effect in dystrophic skeletal muscle.

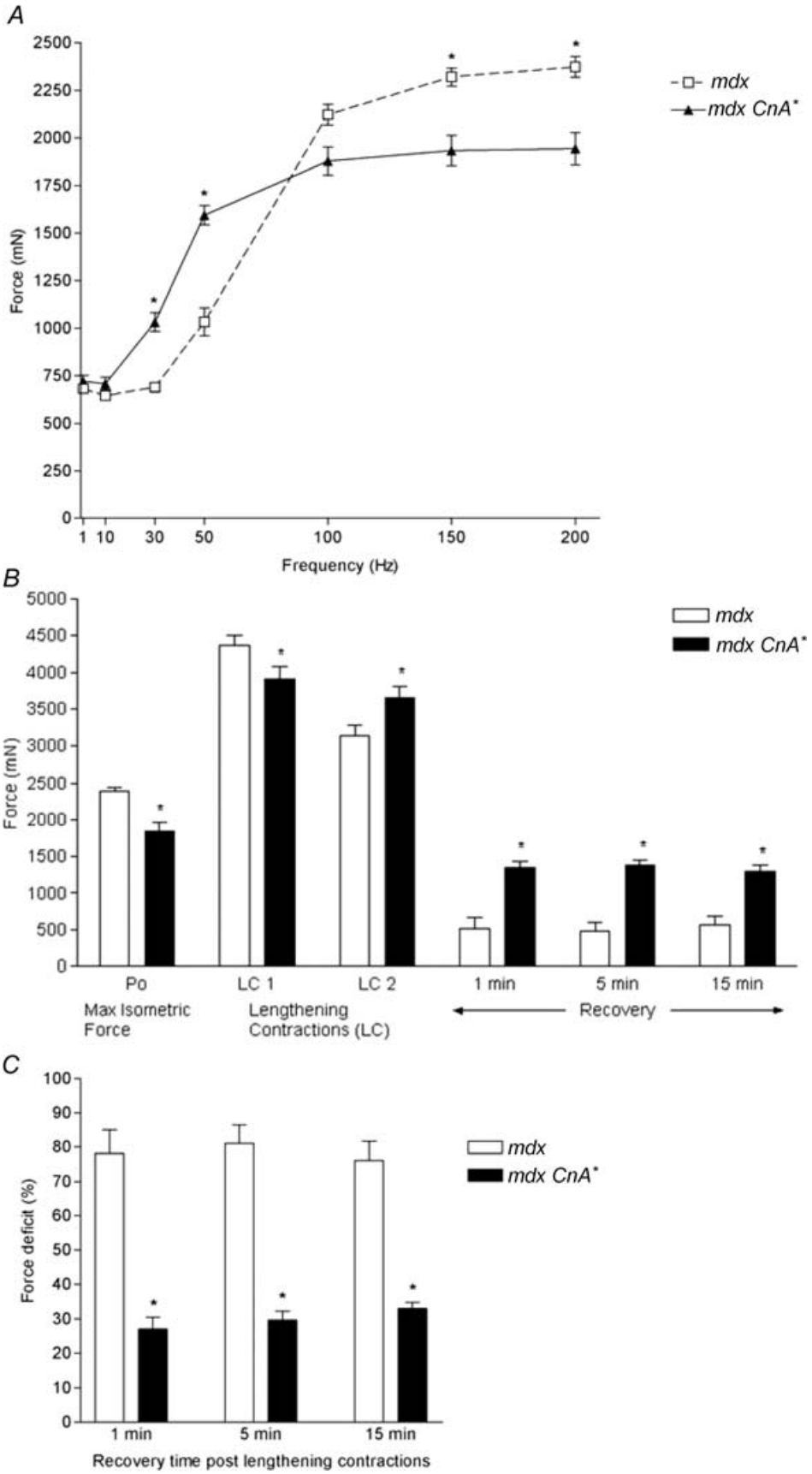
Discussion

We have demonstrated that stimulation of the calcineurin signal transduction pathway can improve dystrophic muscle function by decreasing muscle susceptibility to contraction-induced injury. Expression of the *CnA** transgene in muscles of *mdx* mice attenuated the force deficit of TA muscles following two LCs and reduced muscle membrane damage in both injured and uninjured TA muscles. This protective effect was probably mediated by higher utrophin expression, a decrease in fibre size and a shift towards a slower muscle phenotype. Whereas the effect of increased calcineurin phosphatase activity on muscle structure has been well characterized (Talmadge *et al.* 2004; Naya *et al.* 2000; Chakkalakal *et al.* 2004), its effect on function has not. Compared to muscles from *mdx* mice, TA muscles from *mdx CnA** mice had a higher proportion of fibres expressing the slow(er) MyHC I and IIa isoforms, prolonged TPT and $^1/_2$RT values, lower absolute and normalized maximum forces and a leftward shift of the frequency–force relationship.

Expression of the constitutively active calcineurin transgene has been shown to induce a slower muscle phenotype (Chin *et al.* 1998; Naya *et al.* 2000) and increase utrophin expression at the sarcolemma (Chakkalakal *et al.* 2004), although its effect is muscle specific (Talmadge *et al.* 2004). In both slow-twitch and fast-twitch muscles from MCK-CnA* mice, MyHC type I isoform expression was increased compared to muscle from wild-type mice. Expression of the type IIa MyHC isoform is highly stimulated (100-fold) by calcineurin activation (Allen *et al.* 2001; Allen & Leinwand, 2002), yet it is increased in fast-twitch, but not in slow-twitch muscles from MCK-CnA* mice (Talmadge *et al.* 2004). Others have observed that the increase in type I and IIa MyHC expression in muscles from *mdx CnA** mice is associated with a decrease in type IIb MyHC expression (Chakkal *et al.* 2004).

Our observed differences in utrophin and MyHC I and IIa expression between *mdx CnA** and *mdx* mice were two-fold higher than in previous reports (Chakkalakal *et al.* 2004). This may be attributed to the fact that the basal calcineurin activity levels in the transgenic mice used by Chakkalakal *et al.* (2004) were significantly lower (∼10-fold) than in the mice used in the present study, as reported by Wu *et al.* (2001) and Talmadge *et al.* (2004). In the strain of MCK-CnA* mice used in the present study, the transgene has been reported to represent 21–34% of the endogenous calcineurin pool, although its presence does suppress the expression of endogenous calcineurin (Ryder *et al.* 2004). Furthermore, its gene and protein expression is greater in fast-twitch compared to slow-twitch muscles (Talmadge *et al.* 2004).

The effect of *CnA** transgene expression on muscle mass and fibre cross-sectional area in *mdx* mice has not been reported previously; however, in non-dystrophic (wild-type) fast twitch muscles, expression of the *CnA** transgene decreased both mean fibre cross-sectional area and muscle mass (Talmadge *et al.* 2004). In mammalian skeletal muscle, a strong inverse correlation exists between oxidative capacity and fibre cross-sectional area (Nakatani *et al.* 1999; Talmadge *et al.* 2004). As the decrease in fibre cross-sectional area was four-fold greater than the decrease in muscle mass, we hypothesized that expression of the *CnA** transgene may increase myofibre number in TA muscles of *mdx* mice. Our data support this contention because the number of fibres per mm^2 muscle tissue was almost two-fold greater in TA muscles of *mdx CnA** than in muscles of *mdx* mice. Although our method of evaluating myofibre number has its technical limitations, a similar approach has been used by others to assess changes in myofibre number in response to genetic manipulation of the



calcineurin signal transduction pathway (Parsons *et al.* 2003). The mechanism for the increase in myofibre number in TA muscles of *mdxCnA** mice is unknown. However, muscle fibre number is reduced in soleus muscles of *CnA** knockout mice (Parsons *et al.* 2003), as well as in NFATc3 knockout mice (Kegley *et al.* 2001). It is possible that the constitutively active *CnA** transgene increased NFATc3 activation in TA muscles of *mdxCnA** mice and stimulated hyperplasia.

A large variability in fibre size is characteristic of dystrophic muscles (Bulfield *et al.* 1984; Wehling *et al.* 2001). In this study, expression of the *CnA** transgene did not reduce fibre size variability. We assessed fibre size variability by calculating the CV for each muscle examined; the average CV being ∼70% for *mdx CnA** mice and ∼75% for *mdx* mice. By comparing differences in standard deviation, others have reported a reduction in fibre size variability in muscles of from *mdx CnA* mdx* mice compared to those from *mdx* mice (Chakkalakal *et al.* 2004). However, relying on standard deviation to assess fibre size variability can be misleading. In the present study, the mean standard deviation was 707 μm^2 for *mdx CnA** mice and 1471 μm^2 for *mdx* mice, yet the CV was similar for the two groups.

Expression of the *CnA** transgene improved muscle structure. The proportion of centrally nucleated fibres was reduced by ∼10% in TA muscles from *mdxCnA** mice compared to muscles from *mdx* mice, and this may reflect better myofibre viability (Deconinck *et al.* 1997; Wehling *et al.* 2001; Chakkalakal *et al.* 2004). We postulate that when muscle fibres are less susceptible to damage, there should be a reduced need for regeneration and the proportion of centrally nucleated fibres should decrease. A reduction in the proportion of fibres staining for albumin in uninjured TA muscles in *mdx CnA** mice compared to muscles in *mdx* mice, supports the contention that muscle fibres from *mdxCnA** mice are less susceptible to damage and have improved sarcolemmal integrity. Others have also reported reduced intramuscle fibre staining for Evans Blue, albumin and IgM, as well as lower plasma creatine kinase levels in *mdx CnA** than in *mdx* mice (Chakkalakal *et al.* 2004).

TA muscles from *mdx CnA** mice are more resistant to muscle damage induced by two LCs *in situ*. The force deficit following the injury protocol was ∼30% in muscles from *mdxCnA** mice, but ∼80% in muscles from *mdx* mice. The expected force deficit of TA muscles from adult C57BL/10 (non-dystrophic) mice following the two-LCs protocol has been reported to be ∼15%–25% (Dellorusso *et al.* 2002). The injury protocol increased the number of albumin-positive fibres in TA muscles, but in injured muscles from *mdx* mice, twice as many fibres stained positive for albumin compared to injured muscles from *mdxCnA** mice. Thus, the magnitude of the force deficit and albumin staining suggests that the susceptibility of TA muscles from *mdxCnA** mice to LCs resembles that previously reported for TA muscles of non-dystrophic mice (Dellorusso *et al.* 2002).

Increased utrophin expression can improve the resistance of TA muscles to contraction-induced injury. Expression of truncated (Deconinck *et al.* 1997) or full-length utrophin transgenes (Tinsley *et al.* 1998) or adenoviral utrophin gene transfer (Satoru *et al.* 2000) has been shown to improve dystrophic muscle structure and function. Up-regulation of utrophin was associated with a decrease in central nucleation, indicative of reduced muscle necrosis and regeneration and improved resistance to contraction-induced damage (Deconinck *et al.* 1997; Tinsley *et al.* 1998; Satoru *et al.* 2000). The force deficit and muscle membrane damage after five LCs *in vitro* were similar in EDL muscles from *mdx* mice expressing the full-length utrophin transgene and in C57BL/10 mice, and less than in *mdx* mice (Tinsley *et al.* 1998). The magnitude of muscle membrane damage sustained was also similar in EDL muscles from *mdx* mice expressing full-length utrophin transgene and in C57BL/10 mice, as the proportion of Procion Orange-stained fibres was not different between the two strains. Furthermore, staining was ∼11-fold greater in EDL muscles from *mdx* mice than from *mdx* mice expressing full-length utrophin transgene and from C57BL/10 mice (Tinsley *et al.* 1998). Increased utrophin expression can improve sP_o in muscles from *mdx* mice (Deconinck *et al.* 1997; Tinsley *et al.* 1998; Satoru *et al.* 2000). In contrast, our observation of a decrease in sP_o and P_o of TA muscles from *mdx CnA** mice could be attributed to a reduction in fibre cross-sectional area and a slower muscle phenotype.

The effect of muscle fibre size on muscle susceptibility to contraction-induced injury is controversial. Small fibres may be more resistant to contraction-induced injury than large fibres, because of their greater surface area to volume ratio (Zammit & Partridge, 2005). Extra-ocular

Figure 3. Effect of calcineurin activation on TA muscle function in *mdx* mice
A, frequency–force relationship. Muscles were stimulated at increasing frequencies with 2-min rest between contractions to avoid fatigue. *Significant difference in force output between TA muscles of *mdx* and *mdx CnA** mice ($P < 0.05$). *B*, maximum isometric force (P_o) before lengthening contractions (LCs), force output for LC1 and LC2, and maximum force after the LCs. The muscles were allowed to recover for 1, 5 and 15 min after the LCs, at which times P_o was reassessed (see Methods for details). *Significant difference in force output between TA muscles of *mdx* and *mdx CnA** mice ($P < 0.05$). *C*, relative force deficit following LC. *Significant difference in force deficit between TA muscles of *mdx* and *mdx CnA** mice ($P < 0.05$; strain–stimulation frequency interaction).

muscles have very small fibres and do not exhibit the typical dystrophic pathology (Karpati & Carpenter, 1986). Compared with control soleus muscles of *mdx* mice, fibres from γ-irradiated soleus muscles were smaller and the number of fibres that had not undergone necrosis or regeneration was significantly greater (Granata *et al.* 1998). However, others have suggested that larger muscle fibres may be more resistant to damage during normal activity, because the force required to perform activities of daily living would be a lower proportion of the maximum force-producing capacity (Zammit & Partridge, 2005). Inhibition of myostatin or overexpression of muscle-specific insulin-like growth factor-I (IGF-I) in muscles of *mdx* mice increased fibre size and force producing capacity. Both interventions decreased muscle membrane damage in *mdx* mice, but did not reduce susceptibility to contraction-induced injury (Granata *et al.* 1998; Barton *et al.* 2002; Bogdanovich *et al.* 2002) nor the incidence of centrally nucleated fibres (Bogdanovich *et al.* 2002). It is difficult to elucidate whether the benefits of myostatin blockade and IGF-I over-expression were due to an increase in fibre size or an improvement in muscle regeneration. However, even in muscles from *mdx CnA** mice, where utrophin expression was up-regulated four-fold, it was the larger calibre fibres that were prone to membrane lesions after LCs or in the basal state. The mean cross-sectional area of these fibres was 50%–80% greater than average.

Increased utrophin expression and decreased fibre size may not be the only factors mediating the reduction in muscle damage in the basal state and following LCs in muscles of *mdx CnA** compared with *mdx* mice. Other factors associated with a shift to a slower phenotype may also contribute to the enhanced resistance to muscle damage in *mdx CnA** mice, including greater sarcomere homogeneity and expression of more compliant isoforms of structural proteins (e.g. titin) in slow-twitch compared to fast-twitch fibres (Macpherson *et al.* 1996). Whether such factors are influenced by calcineurin activation remains to be determined; however, rat slow-twitch fibres are less susceptible to contraction-induced injury than fast-twitch fibres at a given percentage of strain beyond

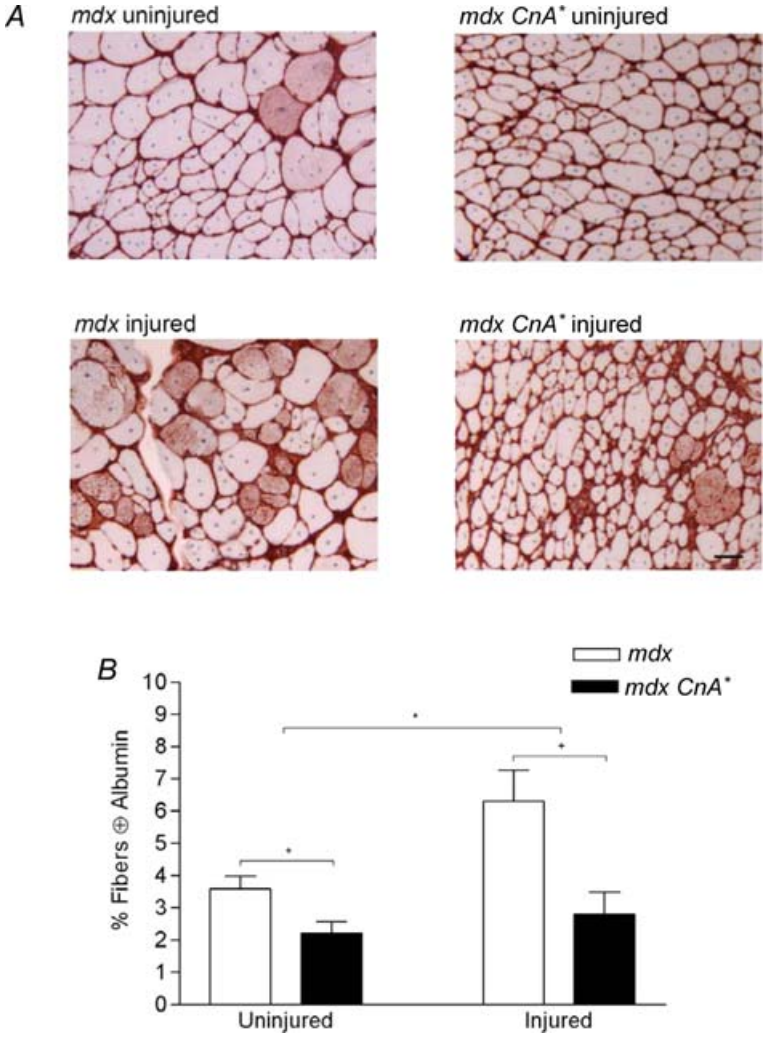


Figure 4. Membrane lesions in uninjured and injured TA muscles of *mdx* and *mdx CnA mice**
A, representative images showing cytoplasmic albumin staining in injured and uninjured TA sections of *mdx* and *mdx CnA** mice. Fibres with membrane lesions stained red; ⊕ = positive. Note that the larger fibres were more susceptible to membrane damage and hence stained positive for albumin. Scale bar represents 100 μm and applies to all panels. *B*, the proportion of albumin-positive fibres in injured and uninjured TA muscles of *mdx* and *mdx CnA** mice. $^{+}$The proportion of albumin-positive fibres was greater in injured than uninjured muscles ($P < 0.05$; main effect); *proportion of albumin-positive fibres was greater in muscles of *mdx* than *mdx CnA** mice ($P < 0.05$; main effect).

optimum length, and slow fibres require a greater strain beyond optimum length for the same force deficit as fast fibres (Macpherson *et al.* 1996). In another study, we found that exogenous administration of IGF-I to *mdx* mice increased succinate dehydrogenase activity, shifted the overall MyHC isoform composition towards a slower phenotype, and of most importance, reduced contraction-induced damage in TA muscles (Schertzer *et al.* 2006).

We have shown that expression of the constitutively active *CnA** transgene in muscles from *mdx* mice not only improves dystrophic muscle structure, as indicated by greater utrophin expression, reduced cytoplasmic albumin staining and fewer centrally nucleated fibres, but also improves function as evident from the lower force deficit after contraction-mediated damage. Although pharmacological interventions that stimulate the calcineurin signal transduction pathway in skeletal muscles may have therapeutic potential for DMD, it is important to note that some studies have implicated increased calcineurin activity in cardiac hypertrophy (Olson & Molkentin, 1999; Zhang, 2002) and aggravated cardiac pathology in *mdx* mice (Nakamura *et al.* 2002). Thus, any therapeutic application that stimulates the calcineurin signal transduction pathway in skeletal muscles would also need to be monitored for cardiac effects very closely.

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