Troponin activator augments muscle force in nemaline myopathy patients with nebulin mutations

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Abstract

Background—Nemaline myopathy—the most common non-dystrophic congenital myopathy—is caused by mutations in thin filament genes, of which the nebulin gene is the most frequently affected one. The nebulin gene codes for the giant sarcomeric protein nebulin, which plays a crucial role in skeletal muscle contractile performance. Muscle weakness is a hallmark feature of nemaline myopathy patients with nebulin mutations, and is caused by changes in contractile protein function, including a lower calcium-sensitivity of force generation. To date no therapy exists to treat muscle weakness in nemaline myopathy. Here, we studied the ability of the novel fast skeletal muscle troponin activator, CK-2066260, to augment force generation at submaximal calcium levels in muscle cells from nemaline myopathy patients with nebulin mutations.

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Methods—Contractile protein function was determined in permeabilised muscle cells isolated from frozen patient biopsies. The effect of 5 μM CK-2066260 on force production was assessed.

Results—Nebulin protein concentrations were severely reduced in muscle cells from these patients compared to controls, while myofibrillar ultrastructure was largely preserved. Both maximal active tension and the calcium-sensitivity of force generation were lower in patients compared to controls. Importantly, CK-2066260 greatly increased the calcium-sensitivity of force generation—without affecting the cooperativity of activation—in patients to levels that exceed those observed in untreated control muscle.

Conclusions—Fast skeletal troponin activation is a therapeutic mechanism to augment contractile protein function in nemaline myopathy patients with nebulin mutations and with other neuromuscular diseases.

INTRODUCTION

Nemaline myopathy (NM) is the most common non-dystrophic congenital myopathy (incidence ~1:50 000).[1] Hallmark features of NM are muscle weakness and the presence of nemaline bodies in skeletal muscle fibres.[2] To date, seven genes have been implicated in NM. Strikingly, six of these genes code for proteins of the skeletal muscle thin filament: α-tropomyosin-3 and β-tropomyosin (*TPM3* and *TPM2*), nebulin (*NEB*), actin α 1 (*ACTA1*), troponin T type 1 (*TNNT1*), and cofilin-2 (*CFL2*). The seventh implicated gene, *KBTBD13*, was recently discovered and the function of its protein product is unknown.[3]

Mutations in the *NEB* gene are the most common cause of NM, likely accounting for >50% of NM cases.[4] Nebulin is a giant sarcomeric protein (~800 kDa); its C-terminus is anchored in the Z-disk and its N-terminus is located close to the thin filament pointed end. Thus, a single nebulin molecule spans nearly the entire length of the thin filament (figure 1). Previous studies of a nebulin knockout mouse model showed that nebulin plays important roles in sarcomeric structure and contractile performance. Nebulin stabilises the thin filament and specifies its length,[5–9] and evidence suggests that it also modulates both the kinetics of actomyosin cross bridge formation[1011] and the calcium-sensitivity of thin filament activation.[11] Recent work from our group revealed that skeletal muscle fibres of NM patients with *NEB* mutations (*NEB*-NM) develop muscle weakness due to loss of these functions of nebulin; their myofibres contain thin filaments of shorter and non-uniform length, they show altered actomyosin cross bridge kinetics,[1213] and they have a lower calcium-sensitivity of force generation.[1314]

To date, no therapy exists that enhances force generation in *NEB*-NM. Strategies to restore thin filament length or the kinetics of actomyosin interaction currently do not exist for skeletal muscle, and, in part due to the extremely large size of the nebulin gene and protein, effective genetic strategies to combat these effects are likely far off in the future. However, the lower calcium-sensitivity of force generation in *NEB*-NM might offer a more immediate therapeutic target, as recently a fast skeletal muscle troponin activator has been developed that amplifies the response of the thin filament to calcium in fast skeletal muscle fibres.[15] The fast skeletal muscle troponin activator *tirasemtiv* (formerly CK-2017357) was shown to

increase greatly the calcium-sensitivity of force generation in healthy rat skeletal muscle fibres,[15] and is currently in phase II clinical studies for amyotrophic lateral sclerosis.[16]

The goal of our study was to test the ability of the novel fast skeletal troponin activator CK-2066260—a close structural analogue of *tirasemtiv*—to improve muscle cell strength in patients with *NEB*-NM. Replication of *tirasemtiv* pharmacology with a different molecule of the same mechanistic class (CK-2066260) strengthens the argument that the described effects are not specific to a single small molecule but instead reflect an effect related to the mechanism of action. Our findings indicate that CK-2066260 greatly increases the calcium-sensitivity of force generation in *NEB*-NM patients to levels that exceed those observed in untreated muscle cells from healthy controls. Thus, fast skeletal troponin activation is a therapeutic mechanism to augment muscle strength in NM patients with nebulin mutations and with other neuromuscular diseases.

METHODS

Muscle biopsies from *NEB*-NM patients

Quadriceps muscle specimens, remaining from diagnostic procedures or obtained during clinically indicated surgical procedures, were collected from four NM patients with confirmed *NEB* gene mutations, from four paediatric control subjects who were biopsied for other diagnostic purposes (with normal skeletal muscle histology), and from three adult control subjects with no medical history. The four biopsies from the *NEB*-NM patients and from the paediatric controls were collected following informed consent supervised by the Boston Children's Hospital Institutional Review Board. The three adult control muscle biopsies were obtained under supervision of the Human Research Ethics Committee, Children's Hospital at Westmead (CHW/10/45). All biopsies were stored frozen and unfixed at −80°C until use. See tables 1 and 2 for details on the clinical and genetic data of the subjects.

Nebulin protein values

To assess nebulin protein concentrations, muscle samples were homogenised and analysed on 1% agarose electrophoresis gels, as previously described.[17] To prevent protein degradation, all buffers contained protease inhibitors (phenylmethylsulfonyl fluoride (PMSF), 0.5 mM; leupeptin, 0.04 mM; E64, 0.01 mM). Gels were scanned and analysed with One-D scan EX (Scanalytics Inc, Rockville, Maryland, USA) software. The integrated optical density of nebulin and myosin heavy chain (MHC) was determined. For western blot analysis, one- or two-colour infrared western blots were scanned (Odyssey Infrared Imaging System, Li-Cor Biosciences, Nebraska, USA) and the images analysed with One-D scan EX.

Pathological evaluation

For electron microscopy, samples were fixed and processed per standard histological techniques for either routine histochemical staining or ultrastructural examination at the time of biopsy, and all slides and ultrastructural images were reviewed by a neuropathologist (MWL).

Muscle mechanics

From the frozen samples, muscle fibre bundles were isolated as described previously.[14] In brief, from the biopsies smaller sections (2×2 mm) were isolated in liquid nitrogen. Subsequently, they were placed for 24 h at −20°C in 4 ml 50% glycerol/relaxing solution containing high concentrations of protease inhibitors (PMSF 0.5 mM, leupeptin 0.04 mM, E64 0.01 mM). Subsequently, the sections were placed on a 'roller band' for 24 h at 4°C, followed by submersing them in skinning solution for 24 h at 4°C. Finally, the skinning solution was substituted with a glycerol/relaxing solution with lower concentrations of protease inhibitors and stored at −20° C until further use. On the day of an experiment, small strips (cross sectional area (CSA) ~0.07 mm^2) were dissected from the glycerinated sections and washed thoroughly with relaxing solution. The strips were mounted using aluminium T clips between a length motor (ASI 403A, Aurora Scientific Inc, Ontario, Canada) and a force transducer element (ASI 315C-I, Aurora Scientific Inc) in a skinned fibre apparatus (ASI 802D, Aurora Scientific Inc) that was mounted on an inverted microscope (Zeiss Axio Observer A1). Sarcomere length was set using a high speed VSL camera and ASI 900B software (Aurora Scientific Inc). Mechanical experiments were performed at a sarcomere length of ~2.1 μm, a length selected to minimise force differences due to shorter thin filaments in fibres from *NEB*-NM patients,[14] and at 2.6 μm to study the effect of sarcomere length on activation. Fibre width and diameter were measured at three points along the fibre and the CSA was determined assuming an elliptical cross section. Fibre dimensions were determined using an inverted microscope and a custom made prism that was mounted in the bath. The dimensions of the muscle strips were measured using a 40× objective and a high speed VSL camera with calibrated ASI 900B software (Aurora Scientific Inc).

Three different types of bathing solutions were used during the experimental protocols: a relaxing solution (40 mM BES; 10 mM; 6.86 mM MgCl$_2$; 5.96 mM Na-ATP; 3.28 mM K-propionate; 33 mM creatine phosphate; 10 mM EGTA DTT; 0.5 mM PMSF; 0.2 mM Leupeptin; 0.05 mM E64; 200 U/ml creatine phosphokinase), a pre-activating solution with low EGTA concentration (40 mM BES; 1 mM EGTA; 6.66 mM MgCl$_2$; 5.98mMNa-ATP; 30.44 mM K-propionate; 33 mM creatine phosphate; 1 mM DTT (dithiothreitol); 0.5 mM PMSF; 0.2 mM leupeptin; 0.05 mM E64; 200 U/ml creatine phosphokinase), and an activating solution (40 mM BES; 10 mM CaCO$_3$-EGTA; 6.64 mM MgCl$_2$; 6.23mM Na-ATP; 2.1 mM K-propionate; 15 mM creatine phosphate; 1 mM DTT; 0.5 mM PMSF; 0.2 mM leupeptin; 0.05 mM E64; 200 U/ml creatine phosphokinase). The temperature of the bathing solutions was kept constant at 15°C using a temperature controller (ASI 825A, Aurora Scientific Inc).

To determine a dose–response curve for the fast skeletal troponin activator CK-2066260 in muscle fibres from controls and from *NEB*-NM patients, tissue was exposed to pCa solutions of 6.0—the pCa that yielded about 35% of maximal active tension—with increasing concentrations (1, 2, 5, 10 and 20 μM) of CK-2066260 dissolved in 1% dimethylsulfoxide (DMSO) as vehicle. Note that 1% DMSO did not affect muscle fibre contractility (data not shown). Figures 5B,C show that CK-2066260 enhanced submaximal force generation with a maximal effect at 5 μM, which is in accordance with previously reported dose–response curves of its analogue CK-2017357.[15] Accordingly, experiments

were conducted with either CK-2066260 (5 μM) or vehicle alone (1% DMSO). To determine the force–pCa relation (pCa=–log of molar free Ca^{2+} concentration), skinned muscle fibre bundles were sequentially bathed in solutions with pCa values ranging from 4.5 to 9.0 and the steady state force was measured (figure 3A). Measured force values were normalised to the maximal force obtained at pCa 4.5. The obtained force–pCa data were fitted to the Hill equation, providing the pCa_{50} and the Hill coefficient, n_H, an index of myofilament cooperativity.

MHC composition of bundles used for contractility experiments

For determination of the MHC isoform composition of the muscle fibre preparations we used specialised SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).[14] In brief, muscles fibres were denatured by boiling for 2 min in SDS sample buffer. The stacking gel contained a 4% acrylamide concentration (pH 6.7), and the separating gel contained 7% acrylamide (pH 8.7) with 30% glycerol (v/v). The gels were run for 24 h at 15°C and a constant voltage of 275 V. Finally, the gels were silver stained, scanned, and analysed with One-D scan EX (Scanalytics Inc, Rockville, Maryland, USA) software.

Statistical analyses

Data are presented as mean±SEM. For statistical analyses, t tests and two way analysis of variance (ANOVA) were used as appropriate. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

Muscle fibres of *NEB*-NM patients have lower nebulin protein concentrations, while myofibrillar ultrastructure is preserved

To study the effect of mutations in *NEB* on its protein product, nebulin protein concentrations were determined in quadriceps muscle of paediatric *NEB*-NM patients and these were compared to those in quadriceps muscle from controls. For all muscle samples, 1% agarose gels showed a single band at the appropriate molecular weight (~800 kDa) of nebulin (figure 2A), which was confirmed to be nebulin by western blot studies using an antibody against nebulin's N-terminus (figure 2B). Quantitative densitometry revealed that both the nebulin/MHC ratio and the nebulin/actin ratio in muscle from paediatric controls ($CTRL_{paediatric}$; 0.051±0.007 for nebulin/MHC and 0.055±0.025 for nebulin/actin, respectively) was not significantly different from the ratio found in adult controls ($CTRL_{adult}$; 0.052±0.003 for nebulin/MHC and 0.040±0.009 for nebulin/actin, respectively). As expected from previous studies,[12–14] muscle from *NEB*-NM patients exhibited a significantly lower nebulin/MHC ratio (0.013±0.003) and a lower nebulin/actin ratio (0.014±0.004) compared to muscle from $CTRL_{paediatric}$ and $CTRL_{adult}$ (figure 2A), indicating that the molecular basis for contractile deficits in muscle fibres from *NEB*-NM patients is related to a relative deficiency of nebulin protein.

Electron micrographs from *NEB*-NM patient muscle biopsies were used to evaluate myofibrillar structure. The organisation of the contractile apparatus was normal in patients with *NEB*-NM (figure 2C), with the exception of areas containing nemaline rods. Thus,

despite the lower nebulin protein concentrations found in muscle from *NEB*-NM patients, their myofibrillar ultrastructure was largely preserved.

The contractile performance of skinned muscle fibres from paediatric controls resembles that of adult controls

Skeletal muscle fibres from *NEB*-NM patients exhibit shorter and non-uniform thin filament lengths, and therefore produce maximal active tension at a sarcomere length of ~2.1 μm.[14] To minimise potential force differences due to shorter thin filaments in *NEB*-NM, most of our studies were carried out at a sarcomere length of 2.1 μm in both control and *NEB*-NM fibres.

Previous work compared contractility of skeletal muscle fibres from paediatric *NEB*-NM patients to that of fibres from adult controls. In addition to adult controls, in the present study we also included a paediatric control group to evaluate the potential confounding effect of age differences on maximal active tension and calcium-sensitivity of force generation.

Figure 3A shows a typical force response of a muscle fibre to various calcium concentrations. From the force–pCa curves, the pCa_{50}—the calcium concentration that produces 50% of maximal force—was determined. The pCa_{50} of quadriceps fibre bundles from paediatric controls was not significantly different from that of slow twitch or fast twitch single fibres from adult controls (5.87 ± 0.02 for $CTRL_{slow}$ (n=6), 5.87 ± 0.01 for $CTRL_{fast}$ (n=23), and 5.88 ± 0.01 for $CTRL_{paediatric}$ (n=15), respectively, figure 3B). As the force–pCa curves indicate, the cooperativity of activation (n_H) in slow twitch (1.52 ± 0.16, n=6) was lower than fast twitch (2.50 ± 0.16, n=23) $CTRL_{adult}$ fibres. Accordingly, the cooperativity of activation in $CTRL_{paediatric}$ bundles (n_H: 2.14 ± 0.09, n=15) was intermediate, but not significantly different from that of $CTRL_{slow}$ and $CTRL_{fast}$, reflecting the mix of slow and fast twitch fibres in the $CTRL_{paediatric}$ bundles.

Maximal active tension was 92.5 ± 9.6 mN/mm^2 in slow twitch $CTRL_{adult}$ fibres (n=6), 107.1 ± 6.4 mN/mm^2 in fast twitch $CTRL_{adult}$ fibres (n=23), and 91.0 ± 11.0 mN/mm^2 in $CTRL_{paediatric}$ bundles (n=15) that contained a mix of slow and fast twitch muscle fibres (figure 3C). Thus, maximal active tension of muscle fibres from paediatric controls was not significantly different from that of adult controls.

These data indicate that the maximal active tension and the calcium-sensitivity of force generation of skinned quadriceps fibres from paediatric controls are comparable to that of adult controls. As it is most ideal to compare data from paediatric *NEB*-NM patients to data obtained from paediatric controls, we limit our further comparisons to these two groups.

The calcium-sensitivity of force generation is lower in skinned muscle fibres from *NEB*-NM patients

The calcium-sensitivity of force generation, as reflected by the pCa_{50}, was lower in quadriceps fibres from *NEB*-NM patients (5.77 ± 0.02, n=20) compared to $CTRL_{paediatric}$ (5.88 ± 0.01, n=15). As shown in figure 4A, the slope of the force–pCa relation was less steep in *NEB*-NM patients compared to $CTRL_{paediatric}$ (n_H: 1.43 ± 0.12 in *NEB*-NM (n=20) vs 2.14

±0.09 in $CTRL_{paediatric}$ (n=15)), indicating that the cooperativity of activation was lower in nebulin deficient muscle. Maximal active tension was also lower in muscle fibres from *NEB*-NM patients compared to $CTRL_{paediatric}$ fibres (15.4±3.5 mN/mm^2, n=20 vs 91.0±11.0 mN/mm^2, n=15, respectively). Thus, the calcium-sensitivity of force generation and maximal active tension were lower in muscle fibres from *NEB*-NM patients compared to those from control subjects.

Since the contractile performance of muscle bundles may be affected by its fibre type composition, we used SDS-PAGE to determine the MHC isoforms of the bundles used for contractile measurements. $CTRL_{paediatric}$ bundles were composed of 46.4 ±5.2% MHC 1, 24.8±5.8% MHC 2A, and 30.7±3.6% MHC 2× (figure 4B). Bundles from *NEB*-NM patients had 63.7±6.7% MHC 1 and 35.6±7.1% MHC 2A. MHC 2× was not observed in bundles from *NEB*-NM patients. The difference in MHC 1 between $CTRL_{paediatric}$ and *NEB*-NM patients had a p value of 0.06 suggesting that there is a trend towards a higher percentage of MHC 1. This skewing to more oxidative fibre types is not surprising, as an increased number of oxidative fibres is often noted in muscle biopsies from patients with NM.[18]

The effect of the fast skeletal muscle troponin activator CK-2066260 on the calcium-sensitivity of force generation

Dose– response relationship—Since troponin activators increase the calcium-sensitivity of force generation, we set out to determine whether the troponin activator CK-2066260 might improve the contractile properties of nebulin deficient muscle. To determine the concentration of CK-2066260 at which it exerts its maximal effect, we first established dose–response curves for the different muscle fibre preparations relating the concentration of drugs to the percentage of maximal force (figure 5). For this purpose, muscle fibres were activated at pCa 6.0—this pCa was chosen because it yielded ~35% of maximal force—in the presence of incremental CK-2066260 concentrations. For a typical response of a fibre to incremental concentrations of CK-2066260, see figure 2A.

Since the bundles of both the *NEB*-NM patients and $CTRL_{paediatric}$ specimens contained a mix of both slow twitch and fast twitch fibres, and because we were also interested in the compound's effect on pure slow twitch and fast twitch fibres, we also tested individual slow and fast twitch fibres from $CTRL_{adult}$. Slow twitch single fibres from $CTRL_{adult}$ (n=2) and *NEB*-NM patient bundles composed of predominantly (>90%) slow twitch fibres (n=2) did not respond to CK-2066260 at any of the concentrations tested (figure 5B,C). In fast twitch single fibres from adult $CTRL_{adult}$ (n=2), mixed $CTRL_{paediatric}$ bundles (n=2), and mixed *NEB*-NM patient bundles (n=3), force enhancement was near maximal at 5 μM CK-2066260 (figure 2B,C). Therefore, a concentration of 5 μM CK-2066260 was used to investigate the effect of the compound on contractile performance of skinned muscle fibres.

Calcium-sensitivity of force generation—The presence of 5 μM CK-2066260 induced a leftward shift in the force–pCa relation in $CTRL_{paediatric}$, as reflected by the increase of the pCa_{50} value from 5.88±0.01 (n=6) in vehicle only to 6.18±0.01 (n=6) in CK-2066260 (figure 6A). The cooperativity of activation was lower in the presence of 5 μM CK-2066260 (n_H 1.70±0.11 vs 2.47±0.13 in vehicle (n=6)). Importantly, in *NEB*-NM patient bundles a

notable leftward shift in the force–pCa relation was observed in the presence of CK-2066260. The pCa_{50} increased from 5.78 ± 0.04 to 6.02 ± 0.02 (n=19), without affecting n_H (1.15 ± 0.11 vs 1.33 ± 0.14 (n=19) in vehicle) (figure 6B). The magnitude of increase in pCa_{50} induced by CK-2066260 was not significantly different in $CTRL_{paediatric}$ fibres (0.26 ± 0.04 pCa units) compared to nebulin deficient NM fibres (0.24 ± 0.05 pCa units). However, when taking into account that the fibre bundles from *NEB*-NM patients contained a higher percentage of non-responsive fibres expressing MHC 1 (figure 4B), the effect size of the compound in fibres expressing MHC 2 might be larger in *NEB*-NM patient fibres. Indeed, a significant correlation was observed between the percentage of fast twitch fibres in the fibre bundles from *NEB*-NM patients and the magnitude of increase in pCa_{50} upon 5 μM CK-2066260 administration (figure 6C, Pearson r=0.79).

Comparing skeletal muscle fibres of *NEB*-NM patients treated with 5 μM CK-2066260 to untreated $CTRL_{paediatric}$ fibres reveals that the fast troponin activator enhances calcium-sensitivity of force generation in *NEB*-NM patients above normal control values (figure 6D). We also investigated whether the maximal active tension was affected by CK-2066260. $Fmax_{CK-2066260}$ expressed as percentage of $Fmax_{vehicle}$ was $96.5\pm1.2\%$ (n=10) in $CTRL_{paediatric}$ and $98.3\pm2.2\%$ (n=18) in *NEB*-NM patients (figure 6E), indicating that CK-2066260 did not affect maximal active tension. Thus, the effect of CK-2066260 is that it completely restores the impaired calcium-sensitivity of force generation in skeletal muscle fibres from *NEB*-NM patients.

The experiments described above were performed at a sarcomere length of 2.1 μm. Because skeletal muscle operates at a range of sarcomere lengths, we also studied a longer sarcomere length (2.6 μm). The calcium-sensitivity of force generation increases when sarcomere length increases (figure 7A), a well known phenomenon called length dependency of activation. Interestingly, this length dependency of activation (expressed as pCa_{50}: the increase in pCa_{50} at a sarcomere length of 2.6 μm compared to that at 2.1 μm) is higher in *NEB*-NM patients (pCa_{50}: 0.15 ± 0.02, n=11) compared to $CTRL_{paediatric}$ (pCa_{50}: 0.090 ± 0.01, n=10) (figure 7A). Due to this more prominent length dependency of activation in fibres from *NEB*-NM patients, the calcium-sensitivity of force generation in these patients (5.88 ± 0.04) was not significantly different from $CTRL_{paediatric}$ (5.97 ± 0.03) (see figure 7B) at a sarcomere length of 2.6 μm. As shown in figure 7B, also at a sarcomere length of 2.6 μm, CK-2066260 induced a pronounced increase in the calcium-sensitivity of force generation in skinned fibre bundles from both $CTRL_{paediatric}$ (pCa_{50} increased from 5.97 ± 0.03 in vehicle to 6.18 ± 0.04 in CK-2066260) and *NEB*-NM patients (pCa_{50} increased from 5.88 ± 0.04 to 6.01 ± 0.06).

For an overview of the pCa_{50} data and for a detailed description of the number of preparations used for each parameters see table 1 of the supplemental data.

DISCUSSION

As lower calcium-sensitivity of force generation contributes to weakness of *NEB*-NM muscle, we studied the ability of the novel fast skeletal muscle troponin activator, CK-2066260, to augment force generation at submaximal levels of activation in skeletal

muscle fibres from *NEB*-NM patients. CK-2066260 greatly increases the calcium-sensitivity of force generation in fast twitch fibres at both sarcomere lengths that were studied to levels that are similar (sarcomere length 2.6 μm) or exceed (sarcomere length 2.1 μm) those observed in control subjects. Thus, fast skeletal troponin activation might be a therapeutic mechanism to augment muscle strength in *NEB*-NM patients.

Force generation is lower in muscle fibres from *NEB*-NM patients compared to those from controls

The severe reductions in force levels generated by fibres of NM patients are clearly due to contractile deficits caused by defects in sarcomeric proteins,[18] rather than lower neural activation, ineffective excitation contraction coupling, or other non-contractile defects. Mechanisms that have been shown to contribute to muscle weakness in *NEB*-NM include myofibrillar disarray,[14 19] shorter thin filaments,[14] altered cross bridge cycling kinetics,[12 13] and a reduced calcium-sensitivity of force generation.[13]

Previous studies indicated that the calcium-sensitivity of force generation is lower in muscle fibres from paediatric *NEB*-NM patients compared to fibres from controls.[13] Because control subjects in prior studies were adults, rather than age matched paediatric control subjects (due to the lack of availability of the latter), it could not be ruled out that the age differences between groups confounded the results. Here, we overcame this limitation by including a control group of paediatric patients, with an average age that was close to that of the paediatric *NEB*-NM patients (table 1), and who were biopsied during investigation of diseases that turned out to be nonneuromuscular in basis and who had histologically normal muscle. Importantly, the contractile performance of muscle fibres from these paediatric controls closely resembles that of adult controls (figure 3B,C), thereby validating our previous work in which paediatric *NEB*-NM patients were compared to adult controls. The *NEB*-NM patients that we investigated in the present study have *NEB* mutations that are distinct from those in our previous work,[13] but their fibres exhibited similar reductions in calcium-sensitivity of force generation (figure 4A), confirming the findings of our previous studies,[13] and suggesting that lower calcium-sensitivity of force generation is a general characteristic in *NEB*-NM patients.

The mechanisms underlying the lower calcium-sensitivity of force generation in *NEB*-NM patients are unclear, but it seems likely that they are a direct consequence of the severely low nebulin protein concentrations that we observed here (figure 2A) and that were also found in our previous work.[12 13] This concept is supported by studies of a nebulin deficient mouse model, which revealed that nebulin augments the calcium-sensitivity of force generation,[11] presumably through its interaction with other regulatory proteins such as tropomyosin and troponin or as a consequence of nebulin's effect on cross bridge cycling kinetics.[20–22] Accordingly, a recent study of muscle fibres from an *NEB*-NM patient with only slightly reduced nebulin protein concentrations showed no change in calcium-sensitivity of force generation.[23]

A novel aspect of the present study was our examination of the length dependency of activation through determination of the calcium-sensitivity of force generation at sarcomere lengths of both 2.1 μm and 2.6 μm. Interestingly, at a sarcomere length of 2.6 μm the

calcium-sensitivity of force generation in muscle fibres from *NEB*-NM patients was not significantly lower than in those from CTRL$_{paediatric}$. Hence, the reduced calcium-sensitivity of force generation contributes to weakness in *NEB*-NM patients mainly when muscles operate at short lengths. The absence of a difference in calcium-sensitivity of force generation at the longer sarcomere length is explained by the exaggerated length dependency of activation in nebulin deficient fibres from NM patients (as shown in figure 7A). These findings are consistent with previous studies on nebulin deficient mouse muscle, which also revealed a lower calcium-sensitivity of force generation compared to that of wild type muscle fibres at a sarcomere length of 2.1 μm,[11] but no difference at a longer sarcomere length.[810] Both these findings and our results imply that nebulin plays a role in the length dependency of activation.[20] This length dependency of activation is most prominent in (nebulin-free) cardiac muscle, where it underlies the Frank–Starling law of the heart, but is much less pronounced in skeletal muscle.[24] The presence of nebulin provides an explanation for why activation in skeletal muscle possesses less length dependency: nebulin increases calcium-sensitivity of force generation at a short sarcomere length. Thus, nebulin deficient muscle from NM patients lacks this feature provided by nebulin, leading to reduced calcium-sensitivity of force generation at short sarcomere lengths.

The novel fast skeletal muscle specific troponin activator CK-2066260: clinical implications for *NEB*-NM patients

To date, no effective therapy is available for *NEB*-NM patients. In the present work, we studied the effect of targeting the lower calcium-sensitivity of force generation in *NEB*-NM muscle fibres by using an analogue of a recently developed compound that amplifies the response of the thin filament to calcium in fast skeletal muscle fibres.[15] The compound that we used structurally and functionally resembles the fast skeletal muscle troponin activator, *tirasemtiv* (formerly CK-2017357).[16] This troponin activator was shown to greatly enhance calcium-sensitivity of force generation in healthy rat and human skeletal muscle fibres[15] and is currently being studied as therapy for amyotrophic lateral sclerosis.[16] *Tirasemtiv* slows the dissociation rate of calcium from the troponin complex,[15] thereby stabilising the open conformation of the troponin/tropomyosin complex to enhance cross-bridge formation at a given calcium concentration. The analogue CK-2066260 is a close structural analogue of *tirasemtiv* and has a similar mechanism of action. Like CK-2017357, CK-2066260 is selective for the fast skeletal troponin complex and has little effect on slow skeletal muscle (figure 5B,C). In the present study, we observed that CK-2066260 greatly increased the calcium-sensitivity of force generation—without affecting n_H—in skeletal muscle fibres from *NEB*-NM patients to levels that exceed those observed in untreated control muscle (figure 6D).

Other troponin activators, such as levosimendan[25] and EMD 57033,[2627] were shown in the past to also increase the calcium-sensitivity of force generation in human skeletal muscle fibres. However, a disadvantage of these agents is that, since they are cardiac/slow skeletal specific, they also affect cardiac function. Increasing the calcium-sensitivity of force generation in cardiac muscle might slow ventricular relaxation which compromises ventricular filling and might cause diastolic dysfunction. Thus, troponin activators that are

specific for fast twitch muscle fibres—such as CK-2066260—are appealing compounds to ameliorate skeletal muscle weakness in skeletal muscle specific myopathies such as NM.

Considering that the normal activation level of muscle is submaximal— between 10% and 65% of its maximal force[28]—during normal activity, the potential of troponin activators for the treatment of muscle weakness in NM patients is high. Our findings reveal that CK-2066260 increases force generation of muscle fibres from *NEB*-NM patients by 20–140% (figure 6B) at the tested submaximal levels of activation. Thus, although the muscle fibres from patients have a diminished capacity to produce maximal force and CK-2066260 did not fully restore maximal muscle fibre force back to control values, the substantial increase in force at submaximal muscle activation produced by CK-2066260 may still improve quality of life in these patients. The benefit from troponin activators involves both increased force development and increased efficiency by reducing the amount of cytosolic calcium that is required to generate a given level of force. Considering that the energy utilisation of the calcium pump SERCA (sarcoendoplasmic reticulum calcium transport ATPase) accounts for 30–40% of total ATP consumption during muscle contraction,[29] the use of a troponin activator has the potential to reduce the amounts of calcium that cycle each contraction, thereby reducing muscle fatigue, which is especially important for the respiratory muscles in patients with NM. Note that respiratory failure, due to diaphragm weakness, is the main cause of death in neonates and children with NM.[2] In support of this potential role for troponin activators in attenuating respiratory failure, a recent study with a cardiac/ slow skeletal specific agent confirmed an improved neuromechanical efficiency and reduced development of fatigue of the human diaphragm during loading tasks in vivo.[30]

It should be noted that the potential therapeutic value of troponin activators such as CK-2066260 is not restricted to NM patients which suffer from a lower calcium-sensitivity of force generation; Russell and coworkers[15] reported force augmentation in the face of limited neural input. In particular, patients with disorders involving defective excitation–contraction coupling such as minicore myopathy, central core myopathy or myotubular myopathy, might benefit by maximising the effect of suboptimal calcium influx. However *NEB*-NM patients might have a larger window for improvement because they suffer from a lower calcium-sensitivity of force generation. Thus, troponin activation is a potential therapeutic mechanism to improve force in NM patients with nebulin mutations as well as potentially with other neuromuscular diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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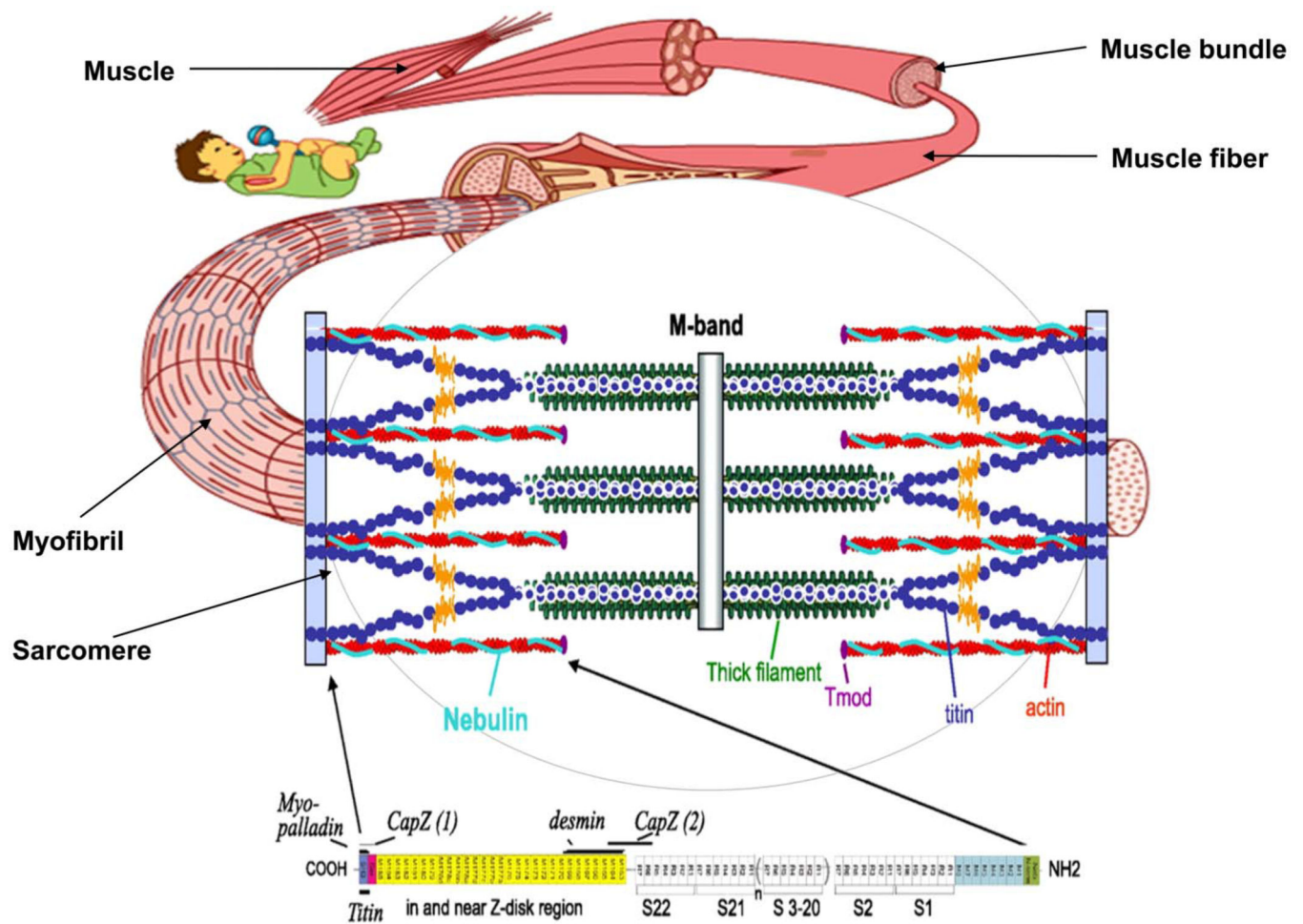
Figure 1.
Schematic of nebulin's location in the skeletal muscle sarcomere. A single nebulin molecule spans the thin filament with its C-terminus anchored in the Z-disk and its N-terminus located close to the thin filament pointed end. The highly modular central region (M9–M162) is divided into seven modular repeats that are arranged into 22 super-repeats.
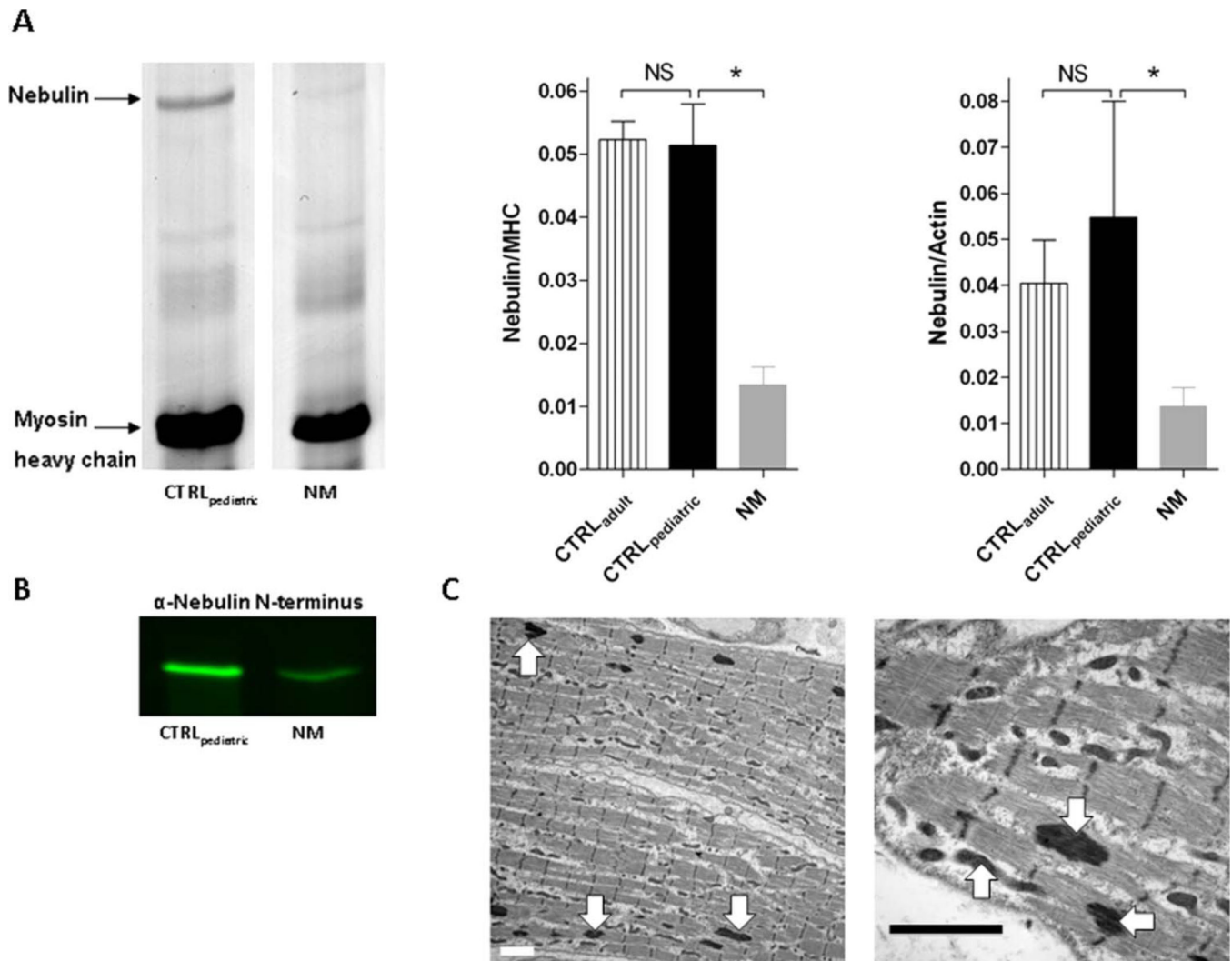
Figure 2.
Nebulin protein values and myofibrillar structure in muscle fibres from nebulin nemaline myopathy (*NEB*-NM) patients. (A) Protein gel example of muscle sample from CTRL$_{paediatric}$ and *NEB*-NM patients (left). Nebulin protein values normalised over myosin heavy chain values were not significantly different between CTRL$_{paediatric}$ and CTRL$_{adult}$, and were significantly lower in muscle from *NEB*NM patients (middle). Nebulin protein values normalised over actin protein values were not significantly different between CTRL$_{paediatric}$ and CTRL$_{adult}$, and were significantly lower in muscle from *NEB*-NM patients (right). (B) Western blot studies using an antibody against nebulin's N-terminus revealed one single band at the appropriate molecular weight (~800 kDa) of nebulin; also note the lower intensity of the nebulin band in the *NEB*-NM patient, suggesting nebulin deficiency. (C) Electron microscopy of *NEB*-NM patient revealed the presence of electron dense nemaline rods in muscle fibres (arrows), surrounded by areas of appropriate myofibrillar organisation. Bar=2 μm. CTRL$_{paediatric}$, paediatric controls; NM, nemaline myopathy.
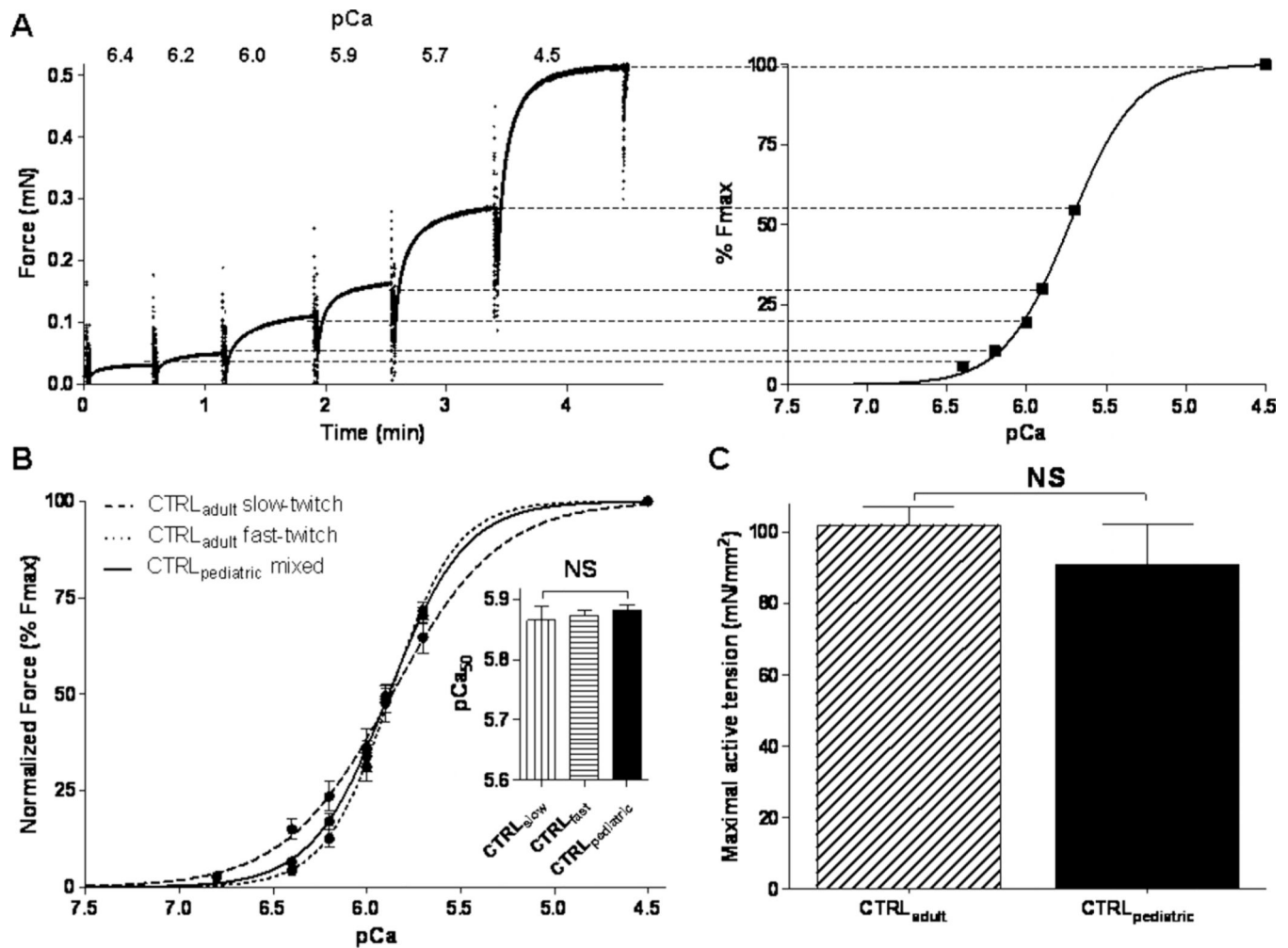
Figure 3.
Maximal active tension and calcium-sensitivity of force generation in skinned muscle fibres from adult and paediatric controls. (A) Example of a force trace from a skinned muscle preparation that is sequentially exposed to incremental Ca^{2+} concentrations (here expressed as pCa: –log of molar free Ca^{2+} concentration). (B) No significant differences in calcium-sensitivity of force generation (reflected by the pCa_{50} value) were found between skeletal muscle bundles from $CTRL_{paediatric}$ and both slow twitch and fast twitch single fibres from $CTRL_{adult}$. (C) No significant difference in maximal active tension was found between skeletal muscle fibres from adult and paediatric controls. $CTRL_{adult}$, adult controls; $CTRL_{paediatric}$, paediatric controls.
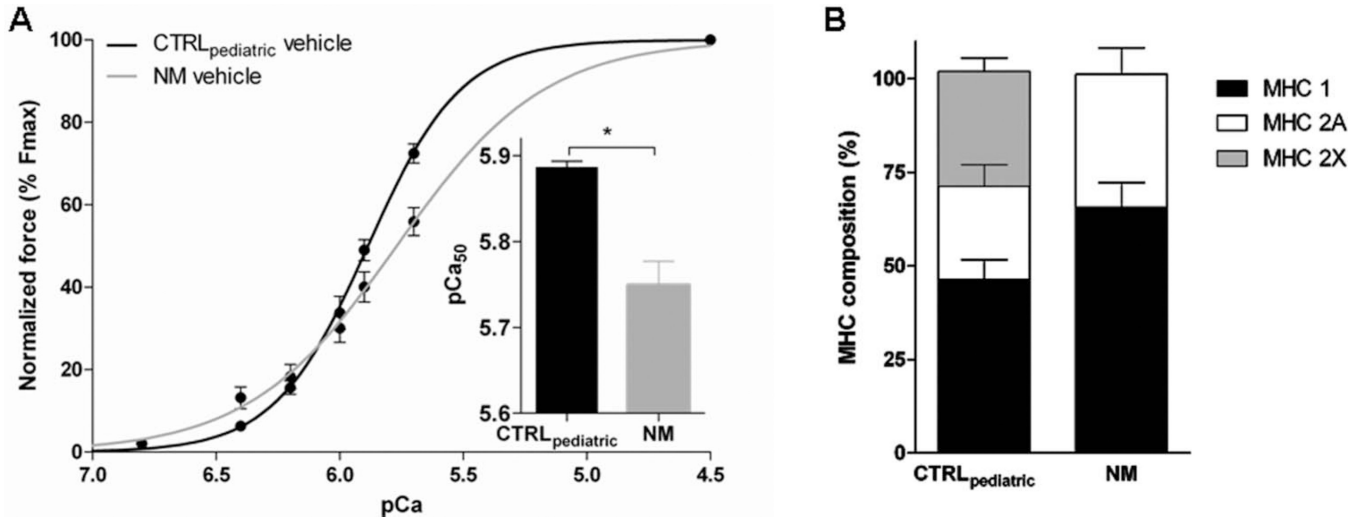
Figure 4.
The calcium-sensitivity of force generation in skinned muscle fibres from nebulin nemaline myopathy (*NEB*-NM) patients and from paediatric controls (CTRL$_{paediatric}$). (A) Calcium-sensitivity of force generation was lower in *NEB*-NM bundles compared to CTRL$_{paediatric}$ bundles ($p<0.05$). (B) Myosin heavy chain (MHC) composition analyses revealed a trend to more MHC 1 in *NEB*-NM bundles compared to CTRL$_{paediatric}$ bundles ($p=0.06$). In addition, bundles from *NEB*-NM patients lack the MHC 2× isoform.
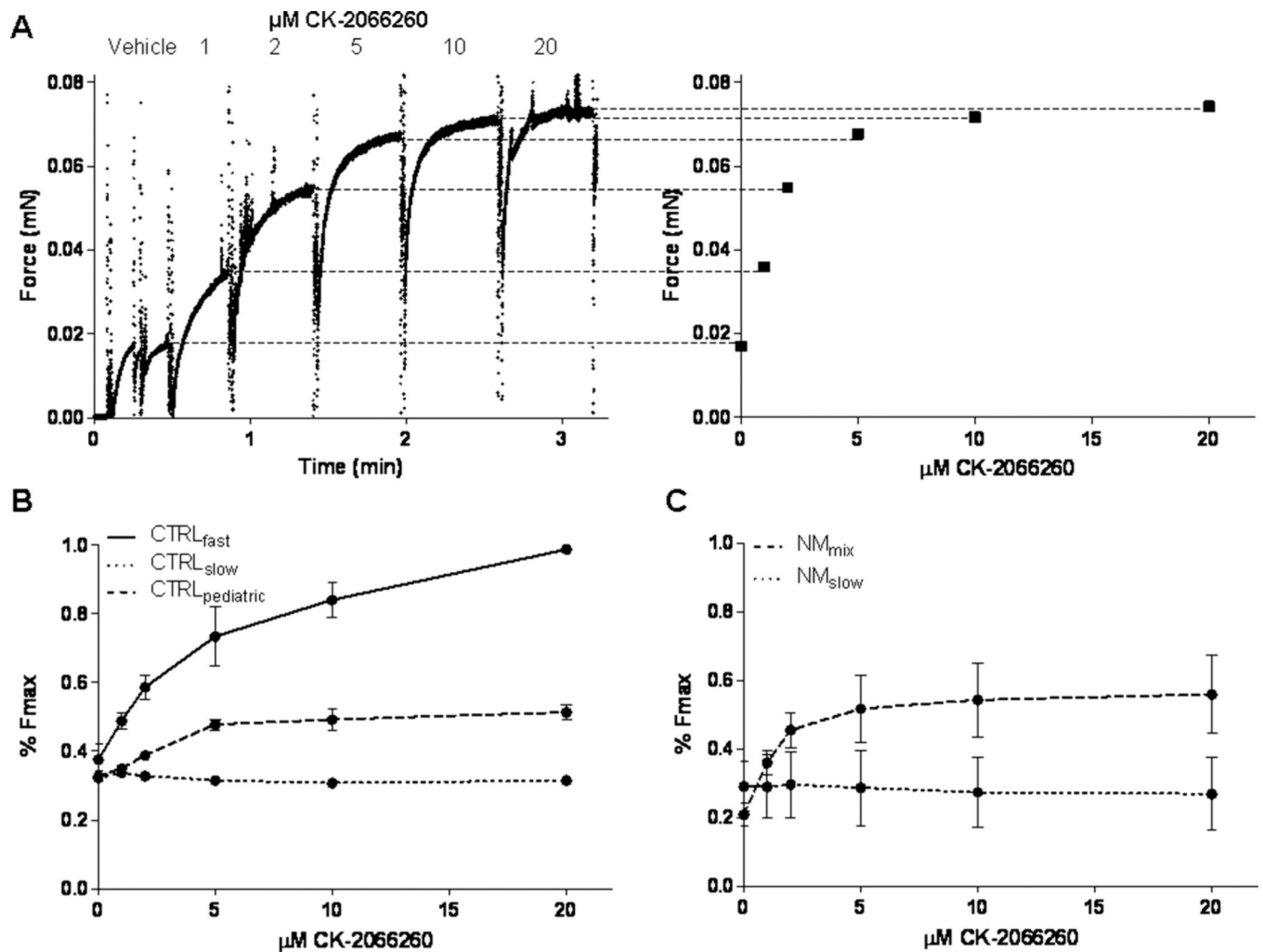
Figure 5.
Dose–response relationship of CK-2066260 in skinned muscle preparations. (A) Example of a force trace from a skinned muscle bundle that is sequentially exposed to incremental CK-2066260 concentrations. (B) Dose–response relation of CK-2066260 in fast twitch and slow twitch $CTRL_{adult}$ single fibres and $CTRL_{paediatric}$ bundles with a mixed myosin heavy chain (MHC) composition. (C) Dose–response relationship of CK-2066260 in predominantly slow twitch (<12% fast twitch) and *NEB*-NM (nebulin nemaline myopathy) bundles with a mixed MHC composition. Vehicle (1% dimethylsulfoxide) was present in all experiments. $CTRL_{adult}$, adult controls; $CTRL_{paediatric}$, paediatric controls; NM, nemaline myopathy.
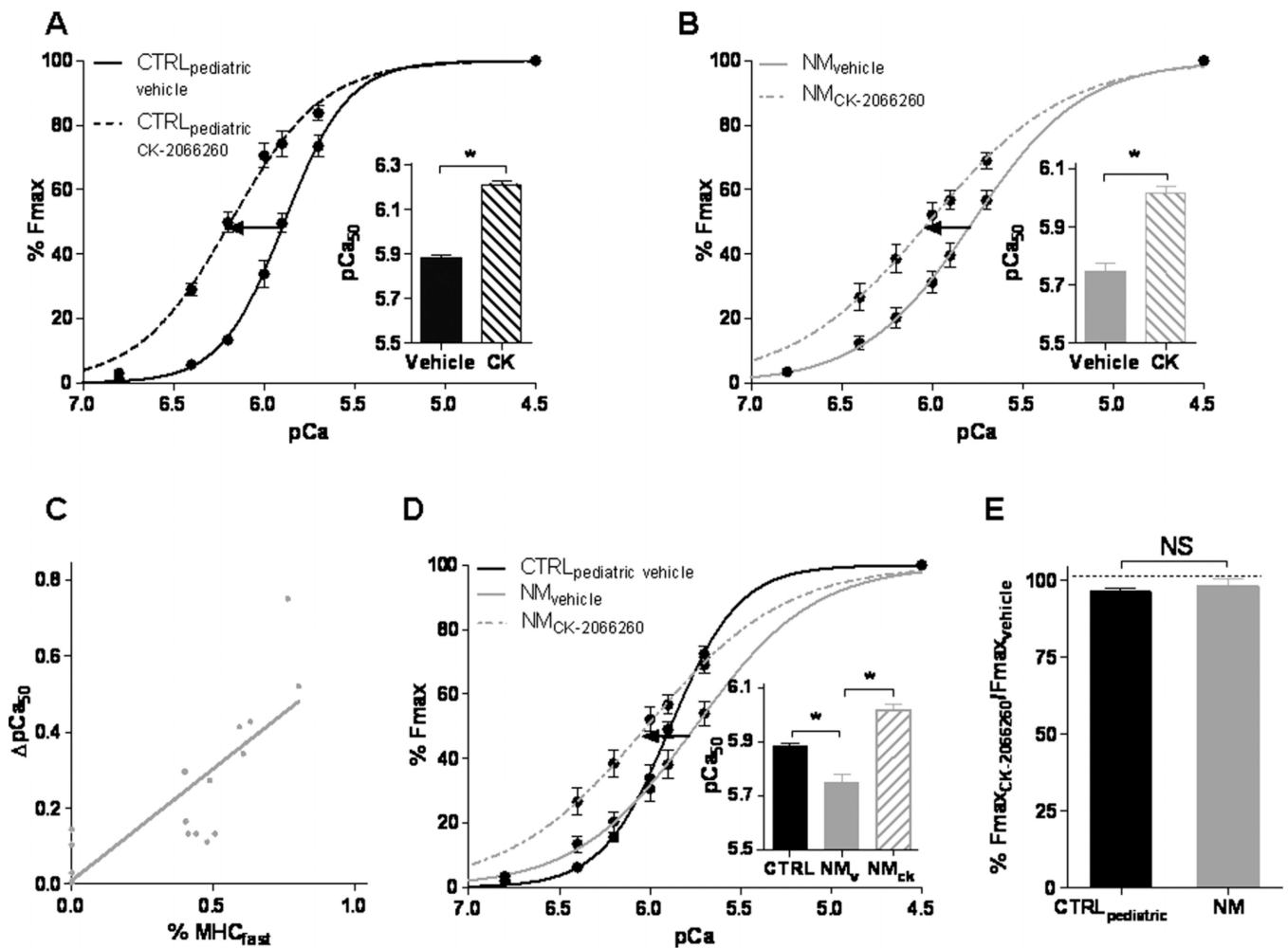
Figure 6.
Effect of CK-2066260 on calcium-sensitivity of force generation in skinned muscle bundles at a sarcomere length of 2.1 μm. (A) CK-2066260 (5 μM) increased the calcium-sensitivity of force generation (reflected by the pCa_{50} value) in muscle fibres from $CTRL_{paediatric}$ ($p<0.05$). (B) 5 μM CK-2066260 increased calcium-sensitivity of force generation in muscle fibres from nebulin nemaline myopathy (*NEB*-NM) patients ($p<0.05$). (C) The CK-2066260 induced shift on the pCa_{50} value was correlated to the NM bundle's MHC_{fast} content (Pearson r=0.79). (D) In the presence of 5 μM CK-2066260, the pCa_{50} of *NEB*-NM patients exceeded that of normal $CTRL_{paediatric}$ values. (E) No significant effect of CK-2066260 on maximal active tension in muscle fibres from $CTRL_{paediatric}$ and *NEB*-NM patients was observed. $CTRL_{paediatric}$, paediatric controls; MHC, myosin heavy chain; NM, nemaline myopathy.
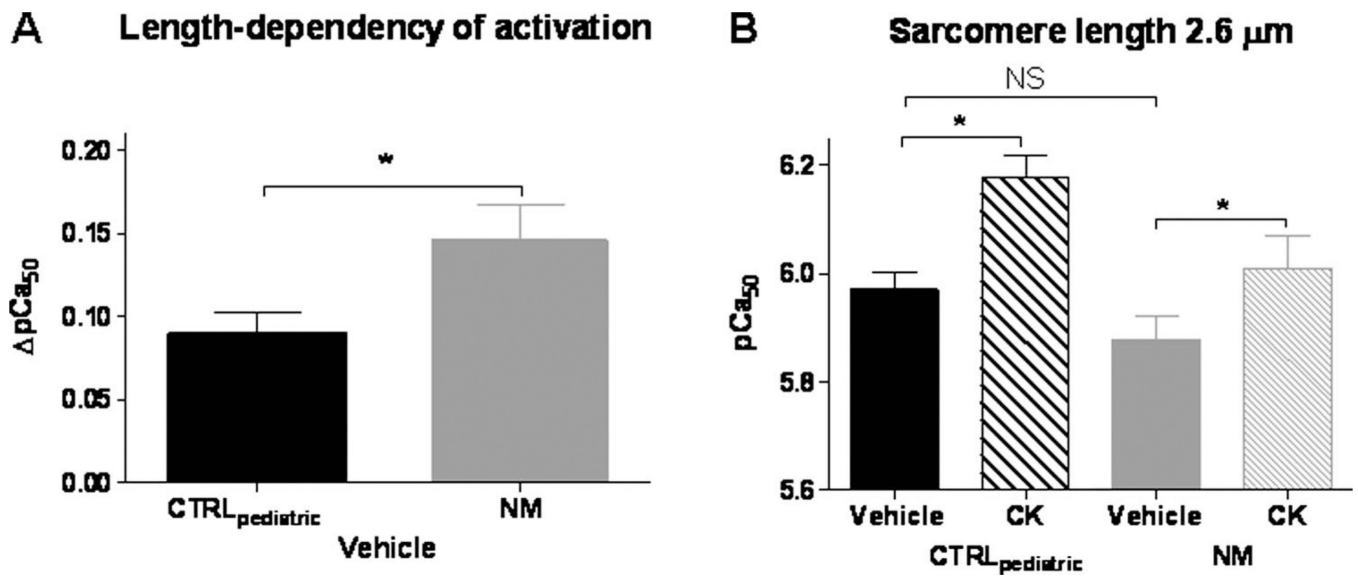
Figure 7.
The calcium-sensitivity of force generation and the effect of CK-2066260 at a sarcomere length of 2.6 μm. (A) Length dependency of activation (expressed as   pCa$_{50}$: the increase in pCa$_{50}$ at a sarcomere length of 2.6 μm compared to that at 2.1 μm) was more prominent in nebulin nemaline myopathy (*NEB*-NM) patients compared to CTRL$_{paediatric}$ (p<0.05). (B) At a sarcomere length of 2.6 μm, 5 μM CK-2066260 increased the pCa$_{50}$ significantly in both muscle fibres from CTRL$_{paediatric}$ and *NEB*-NM patients (p<0.05). At this longer sarcomere length, no significant difference in pCa$_{50}$ between CTRL$_{paediatric}$ fibres and *NEB*-NM fibres was observed. CTRL$_{paediatric}$, paediatric controls; NM, nemaline myopathy.

Table 1

Clinical and pathological data of nemaline myopathy patients and characteristics of control subjects

Patient ID	Biopsy ID	Gender	Biopsy location	Age at biopsy	Fibre typing	Rod characteristics	Clinical form	Age of onset	Maximal motor ability	Clinical status
Nebulin based NM patients										
26–2	T33	Female	Quadriceps	13 months	Extreme type 1 predominance	Numerous large rods in type 1 fibres	Intermediate	Birth	Sat, but never walked	Non-ambulant, requires ventilation and G-tube feedings at 13 years old
258–2	T1069	Male	Quadriceps	4 months	60–70% type 1 fibres, most smaller than type 2s	Subsarcolemmal rods in all fibre types	Typical	Birth	Ambulatory since 18 months	Ambulatory at 5 years old, requires G-tube and BIPAP at night
974–1	T1033	Male	Unspecified	2 months	Hypotrophic type 1, no fibre type predominance	Rods predominantly in hypotrophic type 1 fibres	Intermediate	Birth	Rolling over	Unable to sit at 2 years old, requires G-tube, breathes independently
988–1	T887	Male	Quadriceps	2.5 months	Normal fibre type proportions with significant fibre size variation	Subsarcolemmal rods in all fibre types	Indeterminate	Birth	n/a	Severely hypotonic, requires G-tube and nocturnal ventilation at 5 months
Infant control subjects										
212–1	T141	Male	Quadriceps	3 years	Normal	n/a	n/a	n/a	Normal	Unaffected
213–1	T142	Female	Quadriceps	2 years	Normal	n/a	n/a	n/a	Normal	Unaffected
218–1	T147	Female	Quadriceps	4 years	Normal	n/a	n/a	n/a	Normal	Unaffected
219–1	T148	Female	Quadriceps	5 years	Normal	n/a	n/a	n/a	Normal	Unaffected
Adult control subjects										
n/a	C1	Male	Quadriceps	20	Normal	n/a	n/a	n/a	Normal	Unaffected
n/a	C2	Male	Quadriceps	25	Normal	n/a	n/a	n/a	Normal	Unaffected
n/a	C3	Male	Quadriceps	30	Normal	n/a	n/a	n/a	Normal	Unaffected

BIPAP, bi-level positive airways pressure; NM, nemaline myopathy

Table 2

Genetic data of nemaline myopathy patients

Patient ID	Biopsy ID	*NEB* mutations	Nebulin defects
26–2	T33	c.[7431+1916_7536+372del]+[7431+1916_7536+372del]	p.[Arg2478_Asp2512del]+[Arg2478_Asp2512del]
258–2	T1069	c.[3567+3_3567+7delAAGT]+[18124C>T]	Exon 33 splice defect+p.Gly6041Stop
974–1	T1033	c.[7431+1916_7536+372del]+[24842_24841delAG]	p.[Arg2478_Asp2512del]+[Arg8280SerfsStop2]
988–1	T887	c.[1152+1G>A]+[17013+1G>T]	Exon 13 splice defect+exon 107 splice defect

Pt 26–2 is homozygous for del exon 55.

Pt 258–2 is compound heterozygous for exon 33 splice site mutation and exon 114 nonsense mutation.

Pt 974–1 is compound heterozygous for del exon 55 and 2 bp deletion in exon 177.

Pt 988–1 is compound heterozygous for two splice site mutations in intron 13 and 107.

Reference sequences are NM_001164507 and NP_004534.2.