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Impaired neuromuscular transmission and response to acetylcholinesterase inhibitors in centronuclear myopathies

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Abstract

Many clinical features of autosomal centronuclear myopathies (CNM) and X-linked myotubular myopathy (XLMTM) are common to congenital myasthenic syndromes (CMS). We describe three children whose clinical and electrophysiological findings originally suggested CMS, in whom CNM was diagnosed pathologically, though not yet genetically characterised. A fourth case, with XLMTM, also showed electrophysiological features of a neuromuscular transmission defect. Three (including the XLMTM case) showed improved strength with acetylcholinesterase inhibitor treatment. We also studied neuromuscular junction structure and function in the *MTM1* knockdown zebrafish model of XLMTM, demonstrating abnormal neuromuscular junction organization; anticholinesterase therapy resulted in marked clinical response.

These observations suggest that a neuromuscular transmission defect may accompany CNM and contribute to muscle weakness. Muscle biopsy should be considered in infants suspected to have CMS, especially if treatment response is incomplete, or no CMS gene mutation is identified. Treatment with acetylcholinesterase inhibitors may benefit some CNM patients. This warrants further confirmation.

Keywords: Congenital myasthenic syndromes; Centronuclear myopathy; Neuromuscular junction; Acetylcholinesterase inhibitors

1. Introduction

The centronuclear myopathies are genetically heterogeneous and characterised by prominent, centrally located

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nuclei in a significant proportion of muscle fibres. X-linked myotubular myopathy (XLMTM), caused by mutations in the myotubularin gene (*MTM1*), causes severe weakness in males, usually of prenatal onset with respiratory failure from birth [1,2]. Milder cases and manifesting females have also been reported [3–6]. Autosomal forms are reported with mutations in the dynamin 2 (*DNM2*) [7] amphiphysin 2 (*BIN1*) [8], ryanodine receptor (*RYR1*) [9,10] genes and with inactivating variants in the hJUMPY (*MTMR14*) gene [11]. However, many cases of centronuclear myopathy are genetically unresolved [12].

The *MTM1*, *DNM2* and *BIN1* genes encode proteins with roles in aspects of membrane trafficking and remodelling, although the precise disease pathogenesis in CNM remains to be elucidated [13].

Many clinical features of autosomal CNM and XLMTM are common to other neuromuscular disorders, particularly congenital myasthenic syndromes. We describe three children who, at presentation, had features suggesting congenital myasthenia, including fatiguability which was responsive to acetyl cholinesterase (AChE) inhibitors and abnormal neuromuscular transmission on neurophysiology. However, histopathology suggested a diagnosis of CNM. A fourth case, with molecularly proven *MTM1*-related myotubular myopathy, also had abnormal jitter on stimulation single fibre EMG (stimSFEMG). His fatiguability improved with pyridostigmine. We then demonstrated neuromuscular junction defects in a zebrafish model of XLMTM1, which also responded to AChE inhibitor therapy.

2. Patients

The Dubowitz Neuromuscular Centre is the UK National Commissioning Group referral centre for congenital muscular dystrophies and myopathies, receiving approximately 500 referrals per year. Three children (two girls) were identified between 2000 and 2008 with neonatal weakness, neurophysiological findings suggesting a neuromuscular transmission defect and variable responsiveness to AChE inhibitors, in whom prominent central nuclei were subsequently identified on muscle biopsy, consistent with a diagnosis of CNM. Acetylcholine receptor antibodies were negative. The underlying genetic defect in each case has not yet been identified. Mutations in *MTM1*, *DNM2*, *BIN1, RYR1*, *DMPK*, *TPM2*, *TPM3* and *DOK7* were excluded (Table 1). A fourth case, with molecularly proven *MTM1* CNM, was subsequently studied with stimSFEMG. He had prominent symptoms of fatigue, which improved on treatment with pyridostigmine.

3. Methods

Neurophysiological examination followed standard protocols [14]. Repetitive nerve stimulation (RNS) was considered abnormal if there was greater than 10% decrement in amplitude between the first and fourth response to stimulation at 3 Hz. Stimulation single fibre EMG (StimSFEMG) was considered abnormal in orbicularis oculi if the grand average of the mean consecutive difference (MCD) of the intervals between the stimulus and the potential (jitter) was greater than 32 μs, or with more than 10% of the individual measurements being greater than 44 μs. For extensor digitorum communis (EDC) the upper limits of normal for the MCD were 28 and 40 μs, respectively.

Table 1
Summary of investigations in cases 1–4.

	Case 1	Case 2	Case 3	Case 4
Decrement of RNS	None	30%	None	ND
StimSFEMG	Abnormal	ND	Abnormal	Abnormal
Response to AChE inhibitors	+	+	±	+
Pyridostigmine dependence	+	–	–	+
AChR antibodies	Negative	Negative	Negative	Negative
MTM1 mutation	–	–	–	p.His232Arg
DNM2 mutation	–	–	–	ND
BIN1 mutation	–	–	–	ND
RYR1 mutation	–	–	–	ND
DMPK CTG expansion	–	–	–	ND
DOK7 mutation	–	–	–	ND
TPM3 mutation	–	–	–	ND
TPM2 mutation	–	–	–	ND

Key: + present, – absent, ND not done.

3.1. Zebrafish studies

We previously demonstrated that morpholino knockdown of zebrafish myotubularin faithfully recapitulates the clinical and histopathologic aspects of myotubular myopathy [15]. Using this hypomorphic morpholino model we studied the effect of myotubularin knockdown on the neuromuscular junction. Neuromuscular junction organization was studied by staining 48 h post fertilization (hpf) embryos with Alexa 594 conjugated α-bungarotoxin (Invitrogen). Embryos were photographed using a Nikon Macroscope AZ100. To test the effect of neuromuscular junction augmentation on motor function in our zebrafish morphants, we treated 72 hpf embryos with 0.2 mg/ml edrophonium diluted in egg water. Treated embryos were then filmed for 5 min using time lapse microscopy, and data were reviewed by a blinded examiner.

3.2. Clinical case reports

3.2.1. Case 1

This girl had hypotonia, ptosis, facial weakness and feeding difficulties from birth, requiring nasogastric (NG) feeding and gastrostomy at 2 y. She walked at 23 m, maximum walking distance was 500 m at 5 y. She required nocturnal non invasive ventilation (NIV) from 9 y and spinal fusion for progressive thoracolumbar scoliosis at 12 y. Plasma CK was normal.

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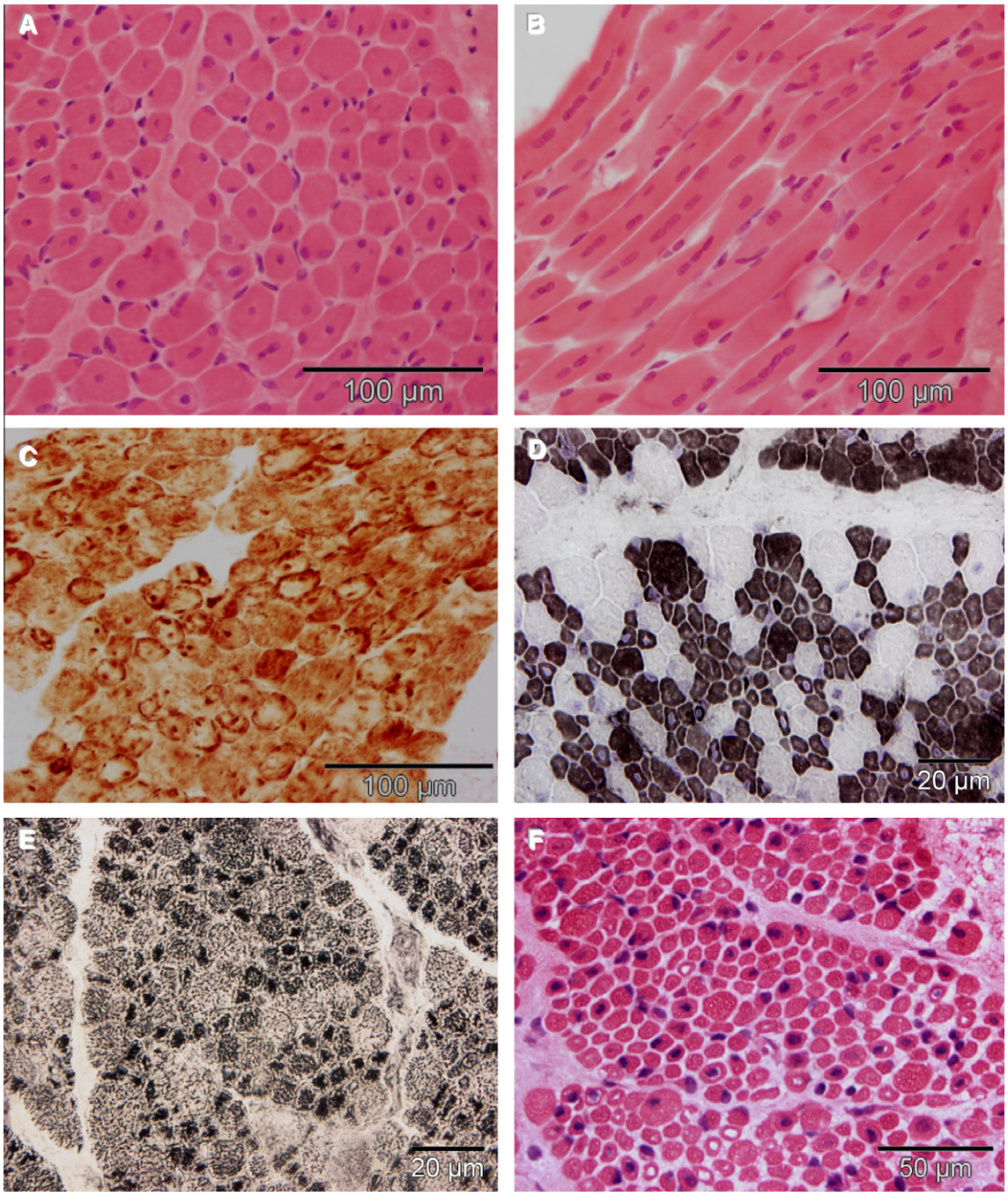

Fig. 1. Muscle biopsy features of cases 1–3. Case 1: (A) H&E showing variation in fibre size with multiple central and internal nuclei, (B) some in chains on longitudinal section, (C) staining for cytochrome oxidase showing central pallor with peripheral aggregation of stain. Case 2: (D) staining for myosin ATPase at pH 4.6 showing several small type 1 fibres with central nuclei and a predominance of type 1 fibres, (E) staining for NADH-TR showing pale peripheral halos around some small type 1 fibres and some fibres that contain a loop of positive staining, as seen in 'necklace fibres'. Case 3: (F) H&E showing variation in fibre size, multiple, prominent central nuclei and several central areas devoid of organelles.

Fatigability was noted from infancy. At 1 y an intravenous edrophonium test was positive and she was treated with oral pyridostigmine, with improved strength. Routine EMG and repetitive nerve stimulation (RNS) of the ulnar nerve at 1, 3 and 5/s were normal, but StimSFEMG (R EDC) showed increased jitter ($n = 8$, mean jitter 58 μs). However open muscle biopsy showed features of CNM with variation in fibre size and many fibres with central or multiple internal nuclei (Fig. 1A). The central nuclei appeared in chains or occasionally separated in longitudinal section (Fig. 1B). There was a mild increase in endomysial connective tissue. With oxidative enzyme stains, the periphery of fibres was frequently darker than the centre which was often pale, resembling cores (Fig. 1C). There were no inclusions, fibre typing was indistinct with all enzyme stains and no radial strands were seen.

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Pyridostigmine was withdrawn, but she became significantly weaker. It was re-started with improvement and continued thereafter. At 16 y, she was ambulant for short distances, with axial and proximal weakness, facial weakness and ptosis (Fig. 2), but normal eye movements.

3.2.2. Case 2

This girl, a non-identical twin born to first cousin parents, had ptosis, hypotonia and stridor from birth. She required CPAP and NG feeds for 3 weeks. At 7 weeks, she had fluctuating ptosis, ophthalmoplegia, a weak cry, facial weakness, truncal hypotonia, mild proximal weakness and hyporeflexia, with oropharyngeal weakness, worsening as feeding progressed. Plasma CK was normal. Neurophysiological examination showed a clear electrodecrement (30%) on RNS (ulnar nerve) at 3 Hz. Oral neostigmine improved strength and feeding times, but recurrent aspiration pneumonia necessitated gastrostomy, fundoplication and cessation of oral feeding at 11 m.

Muscle biopsy at 11 m showed marked variation in fibre size with many small fibres containing central nuclei (Fig. 1D). There was predominance of small type 1 fibres, with larger type 2 fibres, reminiscent of a congenital fibre type disproportion. Oxidative enzymes showed several fibres with dark centres and pale peripheral halos, but no cores (Fig. 1E). A loop of dark oxidative enzyme staining resembling 'necklace fibres' reported in mild *MTM1* [12] cases and one with *DNM2* [16] was seen in some fibres. There were no intracytoplasmic inclusions or radial strands.

Neostigmine was discontinued following the muscle biopsy result. The effect was difficult to judge, as oral feeding had ceased and improved respiratory health enhanced general wellbeing. However, facial weakness, fluctuating ptosis and complete ophthalmoplegia persisted and she remained motor delayed, walking unaided only at 7 y.

3.2.3. Case 3

This boy was born at 37 weeks after polyhydramnios and decreased fetal movements. He was profoundly weak, required resuscitation at birth, had hip and finger contractures, severe facial, bulbar and respiratory muscle weakness and remained ventilated. Seizures occurred on day 1, attributed to birth asphyxia. CK at 3 days was 600 U/l (cardiac massage was performed at birth). At 6 days he remained immobile, nerve conduction studies were normal, EMG of tibialis anterior showed myopathic features and RNS from EDC was normal. However stimSFEMG showed markedly abnormal jitter ($n = 24$, MCD 164 μs, all over 44 μs) (Fig. 3). Pyridostigmine was commenced, with no initial improvement.

Muscle biopsy at 9 days showed abnormal variation in fibre size, prominent central nuclei and central areas devoid of all organelles, appearing as holes (Fig. 1F). Oxidative enzyme stains showed some pale peripheral halos and some central aggregation of stain, but not in all fibres. There were no radial strands. The findings suggested a centronuclear myopathy.

Pyridostigmine was nevertheless continued and he was able to be weaned for periods onto CPAP, but remained intubated with very little spontaneous movement. EEG was low amplitude, with no epileptiform features, brain MRI was normal apart from a small cystic, haemorrhagic

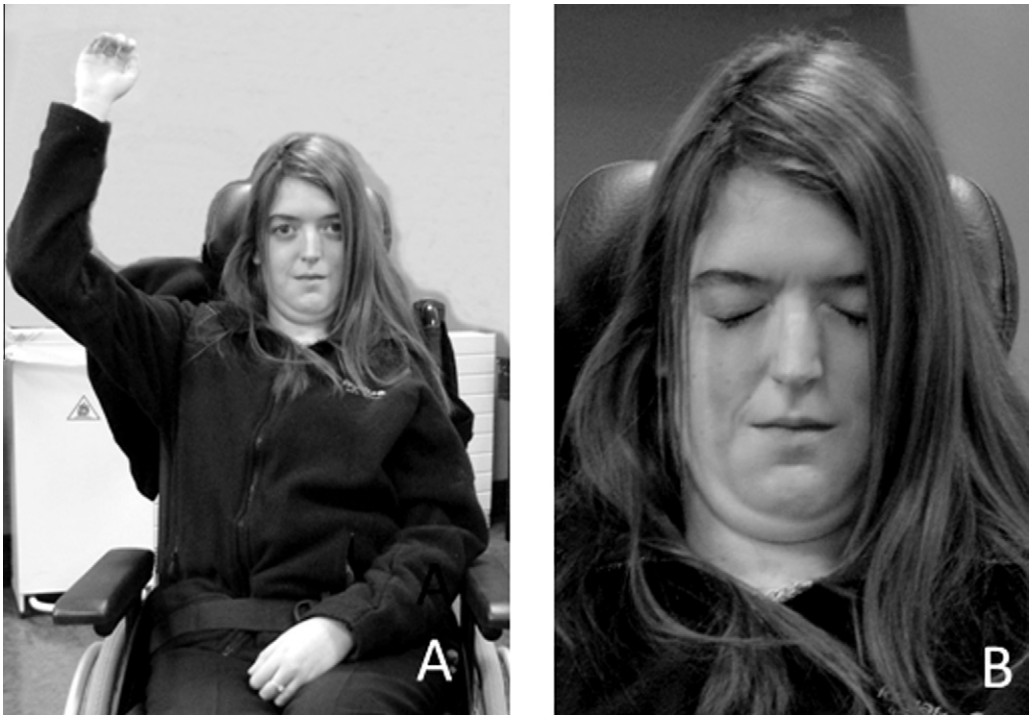

Fig. 2. Patient 1 aged 16 years, showing mild ptosis, facial and arm weakness (A) and weakness of eye closure (B).

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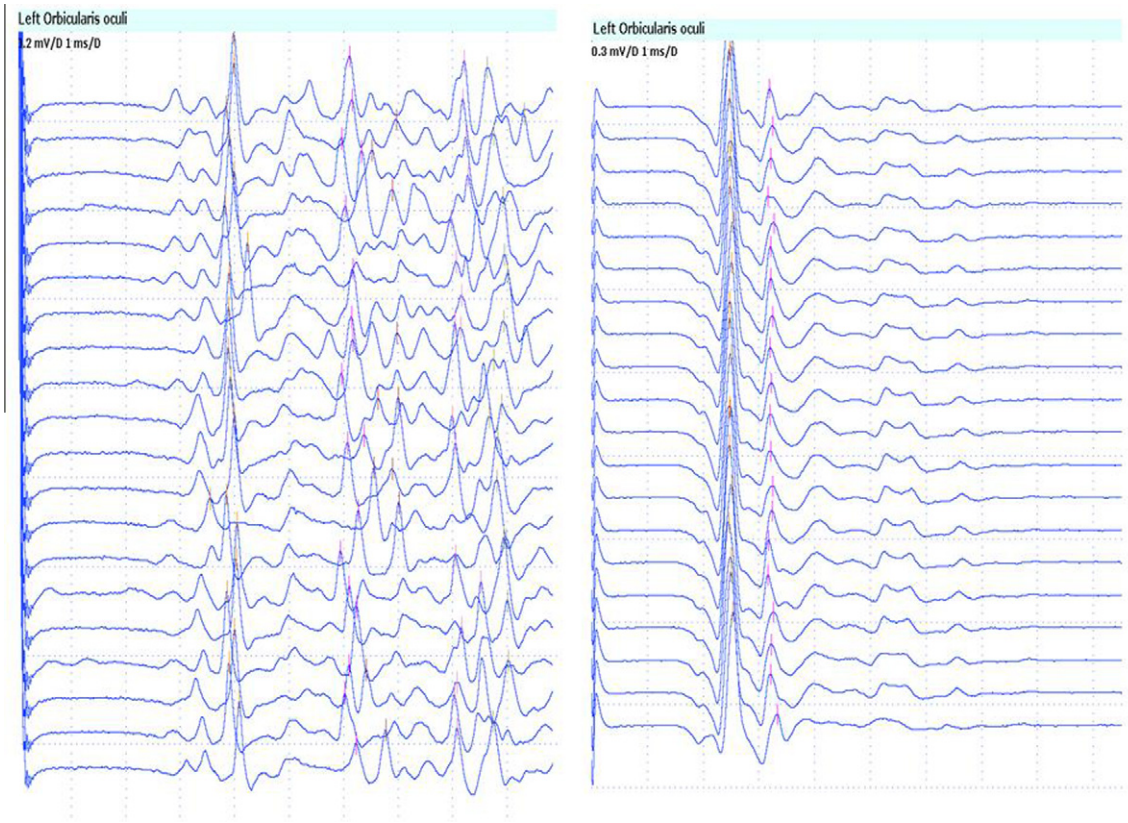

Fig. 3. Stimulation single fibre EMG left orbicularis oculi, Case 3. Results of stimulation single fibre EMG from case 3 (left), and a normal control subject aged two weeks (right) for comparison. Amplification is 0.2 mV per division and 0 .3 mV per division respectively. Time bases: 1 ms per division for both.

ischaemic lesion within the periventricular white matter. Pyridostigmine was eventually stopped because of excessive secretions and he died aged 11 weeks.

3.2.4. Case 4

This boy was born at term, after decreased fetal movements and polyhydramnios. He was hypotonic with facial, axial and limb weakness and undescended right testis. He required NG feeds for 2 weeks, thereafter breast fed with poor suck. He walked at 22 m, but had recurrent chest infections, persistent facial weakness and external ophthalmoplegia. A muscle biopsy performed at another centre at 5 m showed typical features of myotubular myopathy. A missense mutation c.695A > G (p.His232Arg) was identified in Exon 9 of the *MTM1* gene, inherited from his mother. Maximum motor ability was at 5 y when he could walk over a kilometre. Intellect was normal. At 8 y NIV was introduced for 20 min/day, which reduced chest infections and improved pectus excavatum. Motor abilities declined with growth, by 13 y he used a powered wheelchair, walking only one or two steps with support and attended school part time, due to pronounced fatigue. In view of fatigue, stimSFEMG was performed in right orbicularis oculi, which showed mildly increased jitter ($n = 26$, mean jitter 35.8 μs, upper limit of normal 32 μs) with 5/26 > 44 μs (normal less than 10%). Sensory and motor nerve conduction studies were normal, RNS was not performed. A trial of pyridostigmine 30 mg QDS increased his stamina, within days he walked 7 m with assistance, 5–6 times/day. However, following abdominal pain and diarrhoea, pyridostigmine was discontinued, with decline in motor abilities. By age 14 y 3 m, he was unable to weight bear. Gradual re-introduction of pyridostigmine improved stamina and strength once again, allowing him to stand for transfers. His swimming distance improved from 12.5 m before pyridostigmine to 275 m (without stopping) on a daily dose of 45 mg QDS, which he now tolerates well.

3.3. Zebrafish results

We first examined neuromuscular junction organization by staining 48 hpf embryos with Alexa 594 conjugated α-bungarotoxin, which binds acetylcholine receptors at the neuromuscular junction. As depicted in Fig. 4, α-bungarotoxin staining revealed significant changes in neuromuscular junction organisation. Instead of the typical complex clustered pattern of staining seen in control muscle (panel A), staining in myotubularin morphant muscle was attenuated and largely confined to single points in the middle of the myofiber (panels B, C). This abnormal pattern was seen in 90% of embryos examined, compared with 5% of controls ($n = 20$) and persisted at later time points (data not shown). Of note, neuronal input to the neuromuscular junction, as determined by examining α-bungarotoxin

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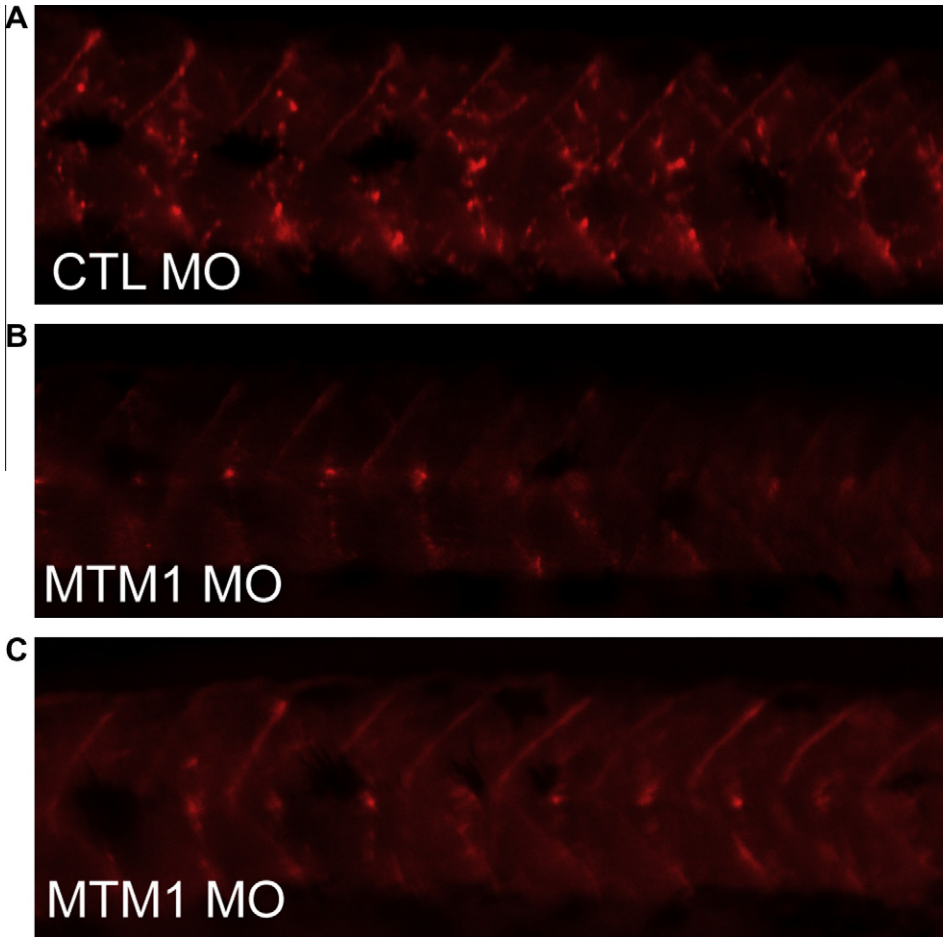

Fig. 4. Neuromuscular Junction Disorganization in a Zebrafish Model of Myotubular Myopathy. 1–4 cell stage zebrafish embryos were injected with equal concentrations of either control (CTL MO) or myotubularin (MTM1 MO) morpholino and allowed to grow until 48 hpf. They were then fixed, processed and stained with Alexa 594 α bungarotoxin. CTL MO embryos showed the expected distribution of α bungarotoxin at this stage of embryonic development [17] (Panel A). MTM1 morphants, on the other hand, had a simplified pattern of staining with abnormal distribution of the acetylcholine receptor (Panels B and C). ($n = 20$ for both conditions).

staining in embryos expressing GFP in their motor neurons, appeared structurally normal.

We then examined the response to neuromuscular transmission augmentation therapy in this zebrafish model of XLMTM. Spontaneous movement and provoked swim behaviour is markedly reduced in the zebrafish knockdown of myotubularin [15] (Supplementary material video 1). Treatment with 0.2 mg/ml of edrophonium resulted in a fast and dramatic improvement in both behaviours in 72 hpf zebrafish injected with myotubularin morpholino ($n = 12$, Supplementary material video 2). Specifically, morphant zebrafish were essentially paralysed prior to treatment (spontaneous movement and touch evoked movement detected in only 1/14 embryos). One minute after treatment, spontaneous tail contractions were observed in 12/14 embryos and 2/14 embryos regained touch-evoked swim response. Maximum response was observed between 3 and 5 min after treatment: 14/14 embryos exhibited spontaneous tail coiling and 5/14 embryos had spontaneous and touch-evoked swim behaviour. No change in response was observed in control embryos: 13/14 embryos exhibited spontaneous coiling and normal touch-evoked swim response both before and after treatment.

4. Discussion

This study reports the presence of a neuromuscular transmission defect in children affected by CNM and the occurrence of a similar phenomenon in the morpholino knockdown of myotubularin in zebrafish. The four children examined illustrate both the variability of clinical presentation and the diagnostic difficulties in CNM. All four had neonatal hypotonia, weakness and feeding difficulties and two required respiratory support. One died in early infancy and three achieved independent ambulation, but two developed increasing motor difficulties in the first decade. All four children had clinical and electrophysiological features suggesting a neuromuscular transmission defect. One showed electrodecrement on RNS at 3 Hz and three had increased jitter on stimSFEMG. Fatigability was a presenting feature in case 1 and a significant finding in cases 2

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and 4. All showed some improvement (albeit limited in case 3) with AChE inhibitors and cases 1 and 4 benefitted from the medication at age 16 and 13 years, respectively. Case 3 had birth asphyxia which may have contributed to his lack of response and poor outcome. The presenting features led to the initial suspicion of a congenital myasthenic syndrome in the first three infants. Mild, non specific histological abnormalities such as variation in fibre size and type 1fibre predominance, features seen in the present cases, occur commonly in the congenital myasthenic syndromes and may lead to an initial misdiagnosis of congenital myopathy [18], but prominent central nuclei are not a feature of CMS histology in childhood. Internal nuclei are found in some patients with *DOK7* mutations [19,20] but they lack the prominent central localisation evident in our cases, which was more characteristic of CNM. The underlying genetic defect has not been identified in three of our cases and further analysis is proceeding. However the finding of impaired neuromuscular transmission in case 4, with a proven *MTM1* mutation might suggest that the genetic basis of these cases is likely to be heterogeneous, as mutations in *MTM1,* as well as *DNM2*, *BIN1* and *RYR1,* have been excluded in the other cases.

Abnormal neuromuscular transmission has been reported before in neurogenic conditions [21,22] and in some primary myopathic conditions [23,24]. However the clinical features (with prominent facial weakness and ptosis) accompanied by the marked neuromuscular transmission defect we documented in our cases, together with the pyridostigmine responsiveness, have not been reported to date in CNM [13,25,26] and may induce diagnostic confusion with CMS. Furthermore, abnormal pre and post synaptic neuromuscular junction organisation has been documented in CNM in the past at the light and electron microscopy level, including paucity of synaptic vesicles, shallow primary clefts and short and unbranched secondary synaptic clefts [27], which could potentially be associated with impaired neuromuscular transmission. The major proteins involved in centronuclear myopathy – myotubularin, dynamin 2 and amphiphysin 2 have all been implicated in membrane trafficking and remodelling [28] and this could potentially give rise to abnormal neuromuscular junction formation and function. There is indeed a known association between membrane trafficking and regulation of the availability of acetylcholine receptors [29].

The possibility of a neuromuscular transmission defect in CNM led us to study the zebrafish morpholino knockdown for myotubularin. In keeping with observations in patients, we demonstrated a significant morphological abnormality of the NMJ and a marked response to edrophonium administration. This and the observed response to AChE inhibitors in our patients suggest that some patients with CNM may benefit from this treatment, although this awaits confirmation. CNM should be considered in infants who present with clinical features of fatigability and/or neurophysiological evidence of myasthenia, especially if their response to pyridostigmine is incomplete and mutations are not identified in the congenital myasthenia genes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2011.02.012.

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