Available online at www.sciencedirect.com

ScienceDirect

Neuromuscular Disorders ■■ (2018) ■■–■■

www.elsevier.com/locate/nmd

Low dystrophin levels are insufficient to normalize the neuromuscular synaptic abnormalities of *mdx* mice

Elizabeth M. van der Pijl [a], Maaike van Putten [b], Erik H. Niks [a], Jan J.G.M. Verschuuren [a], Annemieke Aartsma-Rus [b], Jaap J. Plomp [a,*]

[a] *Department of Neurology, Leiden University Medical Center, Leiden, The Netherlands*
[b] *Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands*

Received 6 October 2017; received in revised form 15 December 2017; accepted 26 February 2018

Abstract

Dystrophin is a sub-sarcolemmal component of skeletal muscle fibres and is enriched at the postsynaptic membrane of the neuromuscular junction (NMJ). In the *mdx* mouse, dystrophin absence not only causes muscle damage but also mild synaptic dysfunctions and clear morphological aberrations at NMJs. In particular, reduction of postsynaptic sensitivity for the neurotransmitter acetylcholine and extra exhaustion of presynaptic acetylcholine release during intense synaptic activity exists. Current experimental therapeutic approaches in Duchenne muscular dystrophy aim to restore dystrophin expression. An important question is what dystrophin levels are needed to improve muscle function. Recent experimental and clinical studies suggested that levels as low as a few percent of normal can be beneficial. Similarly, it is of interest to know how dystrophin levels relate to NMJ function and morphology. We investigated NMJs of a series of *mdx-Xist*^{Ahs} mice, which expressed dystrophin between ~2% and 19% of normal. Most functional and morphological NMJ parameters of these mice remained comparable to *mdx*. On the other hand, *mdx*$^{+/-}$ mice (expressing ~50% dystrophin) showed normal NMJ features. Thus, the minimal dystrophin level required for normal NMJ function and morphology lies between 19% and 50% of normal when expression of dystrophin is not uniform.

Keywords: Acetylcholine receptor; Duchenne muscular dystrophy; Dystrophin; *mdx* mice; Neuromuscular junction; Synaptic transmission

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked myopathy caused by the deficiency of dystrophin, a protein important for the stabilization of skeletal muscle fibres [1]. Dystrophin connects intracellular actin to the dystrophin-associated glycoprotein complex and protects against membrane damage from muscle contraction [2].

Dystrophin is also important in the neuromuscular junction (NMJ), where it is enriched at the postsynaptic membrane [3]. In *mdx* mice, which lack dystrophin and display muscle weakness, NMJs show mild neuromuscular synaptic dysfunction and clear morphological deviations. We and others previously showed reduction of postsynaptic sensitivity for the neurotransmitter acetylcholine (ACh) and extra exhaustion of presynaptic ACh release during intense synaptic activity, causing a reduced safety factor of neuromuscular transmission [4,5]. These functional changes are associated by fragmentation of the postsynaptic ACh receptor (AChR) cluster area [5,6]. DMD patients may have similar NMJ abnormalities, indicated by an increased sensitivity to the AChR blocker d-tubocurarine and the prolonged duration of its effect, as compared to healthy controls [7–11].

Therapies aiming to restore dystrophin expression in DMD are currently under investigation [12,13]. Antisense oligonucleotides have been designed which restore the mRNA reading frame through exon-skipping so that internally deleted, but partly functional dystrophin protein is produced. In this way *mdx* mice have been successfully treated to produce dystrophin with an associated improvement of muscle strength [14,15]. Recent studies in DMD patients suggested slowing of disease progression [16,17]. An exon 51 skipping oligonucleotide has received accelerated approval from the US Food and Drug

* Corresponding author. Leiden University Medical Center, Dept. Neurology and MCB Neurophysiology, Research Building, S5-P, P.O. Box 9600, Leiden 2300 RC, The Netherlands.
E-mail address: j.j.plomp@lumc.nl (J.J. Plomp).

https://doi.org/10.1016/j.nmd.2018.02.013

Please cite this article in press as: Elizabeth M. van der Pijl, et al., Low dystrophin levels are insufficient to normalize the neuromuscular synaptic abnormalities of *mdx* mice, Neuromuscular Disorders (2018), doi: 10.1016/j.nmd.2018.02.013

Administration based on detected dystrophin levels of <1% of normal, although additional studies were requested to confirm functional effects [18].

An important question arising in relation to these therapeutic studies is what level of dystrophin expression is minimally needed to stop muscle degeneration or even restore function. Female DMD carriers, heterozygous for the *DMD* mutation, are mostly asymptomatic and express ~50%–65% of normal dystrophin protein [19–21]. However, some of them express (considerably) less than 50% dystrophin due to skewed X-inactivation and display clear symptoms of muscle weakness [21,22]. This suggests that at least 50% dystrophin is necessary to support muscle function. On the other hand, in exon-skipped *mdx* mice, a dystrophin level of 15% of normal was shown to protect against muscle damage by forced eccentric contractions [23]. Higher levels of 40%–80% dystrophin in addition caused improvement of basal muscle force as assessed in contraction experiments. Other studies in mdx^{3cv} mice and in heterozygous female *mdx* mice with low dystrophin levels due to transgenically induced skewness of X-inactivation revealed that levels of (near full-length or full-length) dystrophin as low as ~5%–15% of normal already improved muscle function [24,25].

In the present study we investigated whether low levels of dystrophin have a beneficial role on the functional and morphological deviations at NMJs of *mdx* mice, which we reported recently [5]. To this end, *mdx* male mice were cross-bred with females homozygous for a mutation in the promoter of the *Xist* gene, which coordinates X-inactivation [26]. Their female *mdx-Xist*$^{\Delta hs}$ offspring expresses variable (but always low) full-length dystrophin levels, as a consequence of preferred inactivation of the wild-type X chromosome [25]. NMJ function and morphology of these mice were assessed and compared to that of heterozygous female *mdx* mice ($mdx^{+/-}$), which express around 50% of normal dystrophin level [27,28], and homozygous female *mdx* mice.

2. Materials and methods

2.1. Animals

Female *mdx-Xist*$^{\Delta hs}$ mice, expressing variable low levels of dystrophin were investigated. They were compared to groups of female *mdx* and $mdx^{+/-}$ mice. The *mdx-Xist*$^{\Delta hs}$ mice were obtained through crossing *mdx* male mice with homozygous *Xist*$^{\Delta hs}$ female mice, which carry a mutation in the *Xist* promoter that coordinates X-inactivation [25,26]. Due to the cross-breeding, *mdx-Xist*$^{\Delta hs}$ mice had a mixed genetic background of the C57BL/10ScSnJ and the *Xist*$^{\Delta hs}$ mice. We also included females of both these control strains in our comparisons in view of possible strain differences in NMJ parameters. C57BL/10ScSnJ and homozygous *Xist*$^{\Delta hs}$ female mice are assumed to express 100% dystrophin. We used mice of 2–5 months of age. All strains studied were bred at the animal facility of the Leiden University Medical Center. Mice were housed in individually ventilated cages at 20.5 °C with 12-h light-dark cycles and had *ad libitum* access to standard RM3 chow (SDS, Essex, UK) and drinking water. All animal experiments were approved by the Animal Ethics Committee of the Leiden University Medical Center.

2.2. In vivo *neuromuscular performance tests*

Respiratory rate and amplitude were assessed with non-invasive whole-body plethysmography in unrestrained animals (RM-80, Columbus Instruments, Columbus, USA). The respiration signal was recorded for 120 s after 30 s acclimatization. The signal was digitized using a Minidigi digitizer and Axoscope 10 software (Axon Instruments/Molecular Devices, Sunnyvale, USA) and analysed with the event detection feature of the Clampfit 10 program (Axon Instruments/Molecular Devices).

The inverted mesh hanging test was used to assess fatigability of limb and abdominal muscles. To this end, mice were placed on an inverted grid forcing mice to hang using all four limbs. The mice were allowed three attempts to hang for a maximum of 240 s. If this maximal hang time was accomplished, the test was stopped. Otherwise the longest hanging time of the three attempts was used for analysis.

Forelimb grip strength was measured with a grip strength meter (type 303500, TSE Systems, Bad Homburg, Germany). Holding the mice at the base of the tail, they were allowed to grasp the pulling bar and were then gently pulled backwards until they released. Ten successive pulls were done with a few seconds pause in between. The mean value was divided by the body weight to obtain the normalized grip strength (g force per g body weight).

Compound muscle action potentials (CMAPs) during repetitive sciatic nerve stimulation-electromyography were recorded from the left calf muscles of ketamine/medetomidine anaesthetized mice as described previously [5].

After completing the electromyography recordings, before recovery from anaesthesia, mice were killed by carbon dioxide inhalation. Diaphragm and epitrochleoanconeus (ETA) muscles were quickly dissected for the electrophysiological and morphological studies described below. Dissected muscles were placed in Ringer's medium at room temperature (20–22 °C), containing (in mM): NaCl 116, KCl 4.5, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 1, $NaHCO_3$ 23, glucose 11, pH 7.4, bubbled with a 95% O_2 and 5% CO_2 gas mixture.

2.3. Ex vivo *neuromuscular junction electrophysiology*

Intracellular recordings of endplate potentials (EPPs) and miniature EPPs (MEPPs) at the NMJ were made in Ringer's solution at 26–28 °C in right phrenic nerve-hemidiaphragm preparations. Muscle fibres were impaled near the NMJ with the tip of a glass micro-electrode (5–20 MΩ, filled with 3 M KCl) connected to a Geneclamp 500B (Axon Instruments/Molecular devices, Sunnyvale, CA, USA) for signal amplification and filtering (10 kHz low pass). In each muscle, 40 muscle fibres were impaled to determine the percentage of NMJs that were synaptically active or 'silent' (i.e. showing no MEPPs for at least one min, and no muscle action potential upon subsequent nerve stimulation). Thereafter, muscle action potentials were eliminated by using the skeletal

Please cite this article in press as: Elizabeth M. van der Pijl, et al., Low dystrophin levels are insufficient to normalize the neuromuscular synaptic abnormalities of *mdx* mice, Neuromuscular Disorders (2018), doi: 10.1016/j.nmd.2018.02.013