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TERMINAL SCHWANN CELL DISTRIBUTION AT THE NEUROMUSCULAR JUNCTION OF THE DYSTROPHIN-DEFICIENT *MDX* MICE

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ABSTRACT

The murine model of muscular dystrophy, the *mdx* mice, is widely used to study the pathogenesis of muscular dystrophies. These mice suffer an X-linked dystrophin deficiency and present cycles of muscle fiber degeneration-regeneration beginning at 21 days of age. At the present, we studied neuromuscular junction organization in the sternomastoid muscle of *mdx* mice, focusing on the distribution of terminal Schwann cells during early development and adults. Seven and 14 days after birth (n=200 endplates for each age), before the onset of muscle degeneration-regeneration, fluorescence confocal microscopy showed that there were no detectable differences in the pattern of Schwann cell distribution in the *mdx* compared to controls of the same age. Schwann cells had a diffuse pattern of distribution, covering the plaques of acetylcholine receptors. In adult *mdx* muscles, terminal Schwann cell processes filled the center of acetylcholine receptors islands, similar to nerve terminal distribution, at the majority of the junctions (n=200; 100%). Conversely, all of the adult control junctions (n=200) showed continuous processes of Schwann cells covering the continuous branches of acetylcholine receptors. These observations indicate that remodeling of the three components of the neuromuscular junction occurs only after the onset of the cycles of muscle fiber degeneration-regeneration, in the *mdx* mice.

Key words: Dystrophy, *mdx*, neuromuscular junction, Schwann cell

INTRODUCTION

The dystrophin-deficient *mdx* mice show a decrease in the sarcolemmal protein dystrophin, a protein in normal skeletal muscle fibers that associates with other molecules of the cytoskeleton to form the dystrophin-glycoprotein complex (DGC; for a review see Rando [20]). These mice arose in the C57Bl/10 colony after a spontaneous mutation in the X chromosome and they show elevated plasma levels of muscle creatine kinase and exhibits histological lesions characteristic of muscle dystrophy [2]. They are the preferred animal model of Duchenne Muscular Dystrophy because of their wide availability and low breeding costs. Experiments in *mdx* mice have provided invaluable information about the importance of dystrophin and its protein complex in the pathogenesis of this disease [3,16].

The molecular organization of the dystrophic neuromuscular junction is of particular interest since it had been suggested that alterations at the neuromuscular junction can also somehow contribute to the pathogenesis of dystrophy [9,24,25], although no changes in neuromuscular transmission have been reported in the *mdx* mice [11]. Several structural abnormalities occur at the neuromuscular junction of *mdx* mice, mainly in the postsynaptic region. These include a reduction in secondary synaptic folding [29], disruption of acetylcholinesterase (AChE) staining [11], changes in the turnover rate of acetylcholine receptors AChRs [30] and in their pattern of distribution [11,15]. In the presynaptic region, the nerve terminals show structural abnormalities associated with the redistribution of AChRs and AChE [11,23]. An increase in the amount of intraterminal sprouting has been seen in *mdx* mice, possibly secondary to muscle fiber regeneration rather than to the absence of dystrophin [23].

Nerve terminal, Schwann cells and acetylcholine receptors are mutually involved in the normal functioning of the neuromuscular junction, and terminal Schwann cells are important for maintaining the structural and functional properties of nerve terminals [17,26,27]. Much is known about nerve terminal and AChR distribution at the dystrophic junction [15,18]. Recently, it had been demonstrated in the diaphragm

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of *mdx* mice that terminal Schwann cells were unable to form bridges between denervated and innervated fibers [19]. At the present we describe the pattern of terminal Schwann cell distribution at the dystrophic neuromuscular junction of *mdx* mice during early development and in adults. We observed that, in the sternomastoid muscle of *mdx* mice, remodeling of the three components of the neuromuscular junction occurs after the onset of the cycles of muscle fiber degeneration and regeneration, typically seen in the adult mice.

MATERIALS AND METHODS

Animals

Mdx and control C57BL/10 mice 7, 14 and 60 days old were obtained from breeding colonies maintained by our animal care facility. Forty two mice (21 *mdx* and 21 controls, 7 mice for each age studied) were used for confocal microscopy. All experiments were done in accordance with the guidelines for the use of animals set forth by our Institution.

Confocal microscopy

Seven, 14 and 60 days after birth (P7, P14 and P60), the mice were anesthetized with a mixture (1:1) of ketamine hydrochloride (ketalar®, Parke Daves) and thiazine hydrochloride (Rompum®, Bayer) in sterile saline solution (2.5 ml/kg body weight, i.p.), perfused intracardiacally with PBS followed by freshly prepared cold fixative (2% paraformaldehyde in PBS). The right and left sternomastoid (STN) muscles were removed, placed in a sylgard dish and washed with PBS. The muscles were then incubated with rhodamine-α-bungarotoxin (Rh-BTX; Molecular Probes, Eugene, Oregon, USA; 1 μg/ml) to label the AChRs and with anti-S-100 (Sigma; Saint Louis, Missouri, USA; 1:500 in blocking solution; n=4 mice for each age) followed by anti-rabbit-IgG-fluorescein (Sigma; Saint Louis, Missouri, USA; 1:500), to stain the Schwann cell or with a mouse monoclonal anti-NF200 (Sigma; 1: 500; n=3 mice for each age), followed by fluorescein-conjugated goat anti-mouse IgG (Sigma, 1:500) to stain nerve terminal. After washing with PBS, the muscles were mounted in 1,4-diazabicyclo [2.2.2] octane (DABCO; mounting medium for fluorescence microscopy) and then observed as whole mounts (without sectioning) using a confocal microscope [12,13].

A dual-channel BioRad laser confocal system (MRC 1024UV) mounted on an Axiovert 100 Zeiss inverted microscope and equipped with Ar-Kr lasers was used. A wavelength of 568 nm was used to excite the rhodamine-labeled receptors and 488 nm was used to excite the fluorescein. The settings for contrast, brightness, and iris diameter were adjusted and kept constant during all observations of control and *mdx* muscles. Optical sections were taken from each endplate, from the top to the bottom and added as a stack of images using the Processing software from BioRad.

Muscle fiber degeneration-regeneration

Seven, 14 and 60 days after birth (5 *mdx* and 5 control for each age), the STNs were removed and stained with hematoxylin and eosin (HE) for evaluation of muscle fiber regeneration, as reported before [14]. Briefly, the mice were anesthetized with an intraperitoneal injection of chloral hydrate (0.6 mg/kg) and perfused intracardially with PBS. The STNs were removed, fixed overnight in 10% formaldehyde, dehydrated through an ethanol series and embedded in paraffin. Transversal sections 6 μm thick were stained with HE and examined with a light microscope (Nikon-Optiphot-2) connected to a Hamamatsu video-camera and a Sony monitor. The number of normal and regenerated (indicated by the presence of central cell nuclei) muscle fibers was counted using a hand counter. No regenerated fibers were seen in the control muscle.

RESULTS

Seven days after birth (P07), AChRs were distributed in plaques at most of the junctions examined in controls (90 ± 10%; Fig. 1A) and *mdx* (78 ±10%; $p>0.05$ compared to control, Student's t-test; Fig. 1C). On P14, most of the controls (75±8%) and dystrophic junctions (90±10%; $p>0.05$ compared to control, Student's t-test) displayed receptors distributed in regular and continuous branches (Fig.1D, F). At adult control junctions (P60), receptors showed the normal pretzel-like pattern of distribution in 100% of the junctions (Fig. 1G), while in adult dystrophic junctions they were distributed in islands (95±5%; Fig. 1I).

S-100 labeling was seen at most of the P07 endplates examined (n=200), with the Schwann cells processes diffusively distributed and clustered over the receptor-rich area. A similar pattern of terminal Schwann cell distribution was seen in dystrophic and controls mice (Fig. 1A, B). Nerve terminals covered the AChRs plaques and displayed bulbous enlargements at their tips (Fig. 1C).

At the branched-junctions of dystrophic P14, Schwann cell presynaptic processes running towards the endplate and covering the receptors were seen (Fig. 1E). The nuclei of terminal Schwann cells

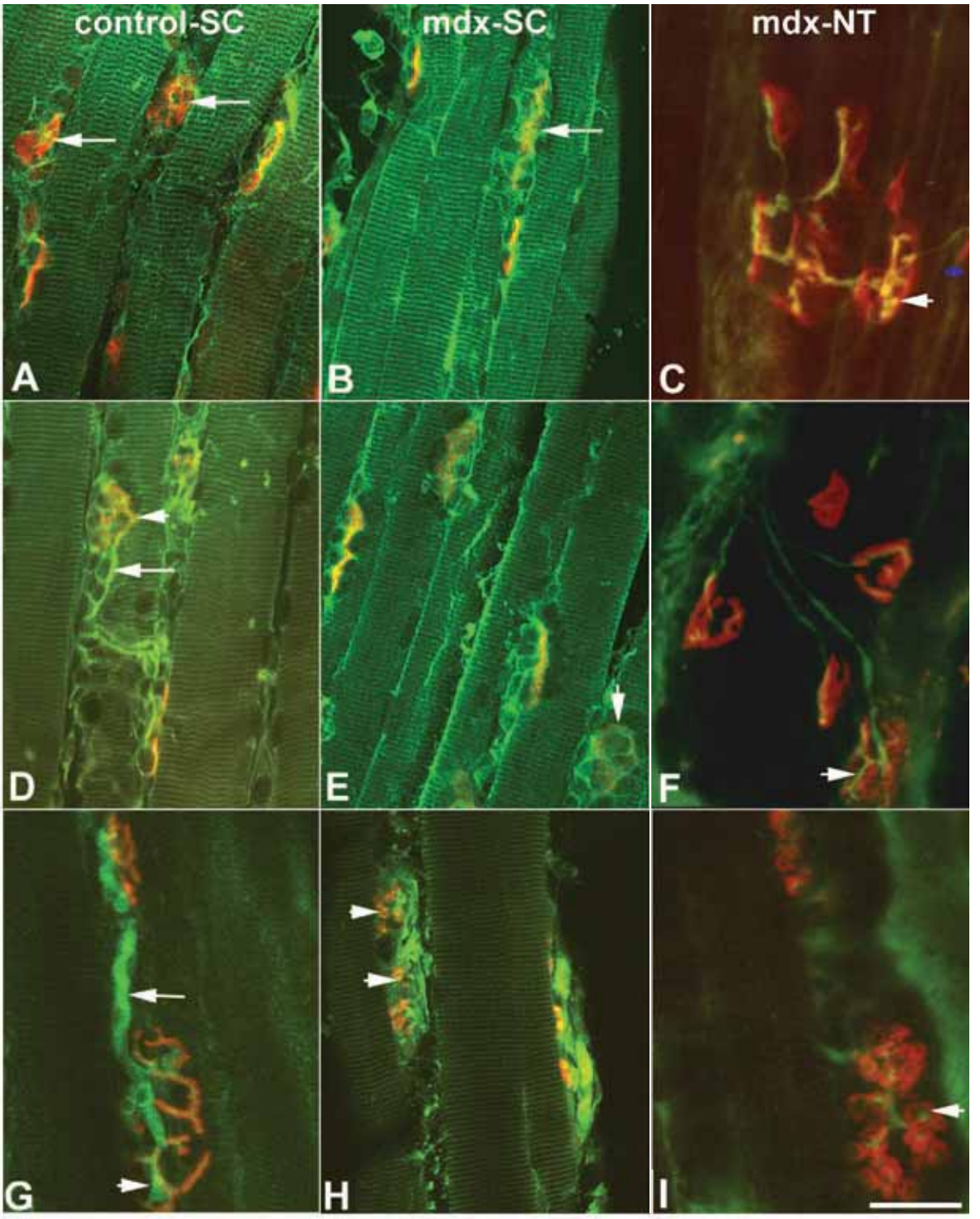

Figure 1. Schwann cell (**SC-green**), acetylcholine receptor (**red**) and nerve terminal (**NT-green**) distribution during postnatal development at control and *mdx* neuromuscular junctions. P07 (**A**, **B**, **C**): Schwann cells with poor cytoplasmic processes are clustered over the AChR rich area (**arrows** in A, B). AChRs (**red**) were distributed in plaques (C). Nerve terminals with bulbous enlargements at their tips (**arrowhead**, C). P14 (**D**, **E**, **F**): a presynaptic process of SC (D, **arrow**) and processes that cover the continuous branches of receptors (D, E, **arrowhead**). Nerve terminals cover the branches of receptors (F, **arrowhead**). P60 (**G**, **H**, **I**): SC main bundle (G, **arrow**) and terminal process over the continuous branches of receptors (G, **arrowhead**). In the *mdx*, spots of SC processes (H, **arrowhead**) inside the islands of receptors, in a pattern similar to the nerve terminal (I, **arrowhead**). Bars: 15 μm (A, B, C), 30 μm (D, G-I), 45 μm (E, F).

were seen as round or elliptical areas framed by thin terminal Schwann cell processes. No processes were seen outside the AChR-rich area. A similar pattern of distribution was observed in control P14 (Fig.1D) junctions, where S-100-positive processes were seen over the AChRs branches, at most of the endplates (n=200 junctions). Nerve terminals covered the branches of receptors (Fig. 1F).

In P60 *mdx* muscles, Schwann cell processes were grouped presynaptically (Fig. 1H). The single, main presynaptic branch usually seen in controls (Fig. 1G) was generally absent in *mdx* junctions. Distally, S-100 labeling was seen as small dots which filled the center of the AChR islands (Fig. 1H). Nerve terminals also filled the center of receptors islands (Fig. 1I).

At most of the control P60 junctions examined (n=200), a major presynaptic prolongation containing the anti-S-100 antibody was seen projecting towards the endplate region (Fig. 1G). Near the endplate, this prolongation branched over the receptors with continuous processes that covered the AChRs.

No signs of muscle regeneration were seen in the early stages of muscle development, indicated by the presence of central cell nuclei. In P07 dystrophic muscles, 0.62% of muscles fibers were regenerated (4 out of 649) and 2.20% (20 out of 920 muscle fibers) of the P14 fibers showed central nucleation. In adult dystrophic muscles, 90% (990 out of 1100 muscle fibers) of the fibers were regenerated ($p<0.05$, significantly different at each time point; Student's t-test).

DISCUSSION

In this work, we analyzed the structural organization of the neuromuscular junction of the dystrophin-deficient *mdx* mice. The *mdx* mice has been extensively used as a model for studying the cascade of events occurring in dystrophin-deficient muscles [16], since it is readily available and offers an economic way for testing possible therapeutic drugs for the dystrophynopathies [3].

Mdx mice show cycles of degeneration-regeneration starting around postnatal day 21 [28]. We observed that, during the earlier stages of postnatal development (P07 and P14), there were no signs of muscle fiber degeneration, indicated by the lack of necrosis, and few regenerated fibers were seen. During these ages, AChRs changed from plaque (P07) to a branched-form (P14), which is in agreement with our previous observations [15]. Nerve terminals also changed from a diffuse (P07) to a branched pattern (P14) and Schwann cells followed the distribution of the nerve terminal. Similar changes in Schwann cell distribution were seen in control muscles, in agreement with previous observations that during normal development, Schwann cells are initially clustered over the endplate area, assuming a branched form typically in adults [7]. Therefore, before the onset of muscle fiber degeneration-regeneration, the pattern of neuromuscular junction organization proceeds normally in the dystrophic muscle.

Adult dystrophic muscles (P60) were fully regenerated and dramatic changes in Schwann cell distribution were seen at this age. The changes paralleled those in nerve terminal architecture reported earlier [11,23], as well as the changes in AChRs reported for adult *mdx* mice [11,15]. The changes in AChRs distribution have been related to muscle fiber regeneration rather than to an absence of dystrophin [14]. Muscle fiber regeneration also appears to be involved in the increased intraterminal sprouting seen in *mdx* mice [23]. Schwann cells can stimulate and guide nerve sprouts in response to a nerve lesion [21,26,27]. As shown here, Schwann cells emitted processes that occupied the center of AChR islands (Fig. 1H). Since nerve intraterminal sprouts occupy the center of the AChR island [23; Fig. 1I), these Schwann cell processes probably accompanied the intraterminal nerve sprouts. Therefore, the three components of the neuromuscular junction changed their pattern of organization, possibly in response to muscle regeneration. It is possible that cell adhesion molecules such as NCAM (neural cell adhesion molecule), which is produced by regenerated muscle fibers [5] and stimulates nerve sprouting [31], also activates terminal Schwann cells.

Studies have suggested that molecular changes at the neuromuscular junction might partialy explain the pathogenesis of DMD [9,24,25,30], although physiological studies of neuromuscular transmission in *mdx* mice showed no changes from normal [11]. Dystrophin plays a key role in the organization of AChRs [10]. Terminal Schwann cells are important for maintaining the structural and functional properties of nerve terminals and these cells respond vigorously to nerve damage [21] in order to improve nerve regeneration and muscle reinnervation [8]. In the diaphragm of *mdx* mice, which seems to be more affected than other muscles, terminal Schwann

cells failed to form bridges between denervated and innervated muscle fibers, which are normally present in this muscle [19]. No denervated muscle fibers were seen in the sternomastoid muscle of *mdx* (14,18,23] or other muscles [29]. At present, we observed that Schwann cells are able to react to muscle regeneration, since their pattern of distribution correlated to that of the nerve terminal and acetylcholine receptors.

Terminal Schwann cells may regulate synaptic function by modulating the production of NO that could act as a local and/or retrograde second messenger [6]. In *mdx* mice, nNOS is dramatically reduced in the sarcolemma of dystrophic muscles [1,4]. nNOS is also dramatically reduced in the presynaptic region of dystrophin-deficient fibers of *mdx* mice, and confocal fluorescence microscopy studies suggest that nNOS is associated with terminal Schwann cells [18,22]. If we consider that NO is a potential messenger for Schwann cell-nerve terminal interactions [6], the lack of nNOS at the presynaptic region might explain the finding that terminal Schwann cells were unable to respond to muscle denervation seen in more affected muscles, such as the diaphragm [19].

In conclusion, these results indicate that remodeling of the three components of the neuromuscular junction occurred after the onset of the cycles of muscle fiber degeneration-regeneration, in the *mdx* mice. In the presence of muscle regeneration, Schwann cells distribution parallels that observed for the nerve terminal and acetylcholine receptors. This finding suggests that these cells are able to react in the absence of a nerve lesion, possibly in response to sprouting factors produced by regenerated muscle fibers. This phenomenon may be of relevance for the reinnervation of new muscle fibers generated by cell-mediated therapies for the treatment of dystrophynopathies and may underlie some of the mechanisms involved in muscular dystrophies in mice and humans.

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