REVIEW

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STRUCTURAL BIOLOGY OF THE DYSTROPHIN-DEFICIENT MUSCLE FIBER

Maria Julia Marques

Department of Anatomy, Institute of Biology, State University of Campinas (UNICAMP), Campinas, SP, Brazil.

ABSTRACT

The discovery of dystrophin and its gene has led to major advances in our understanding of the molecular basis of Duchenne, Becker and other muscular dystrophies related to the dystrophin-associated protein complex. The concept that dystrophin has a mechanical function in stabilizing the muscle fiber membrane has expanded in the last five years. The dystrophin-glycoprotein complex is now considered a multifunctional complex that contains molecules involved in signal transduction cascades important for cell survival. The roles of dystrophin and the dystrophin-glycoprotein complex in positioning and anchoring receptors and ion channels is also important, and much of what is known about these functions is based on studies of the neuromuscular synapse. In this review, we discuss the components and the cellular signaling molecules associated with the dystrophin-glycoprotein complex. We then focus on the molecular organization of the neuromuscular junction and its structural organization in the dystrophin-deficient muscle fibers of *mdx* mice, a well-established experimental model of Duchenne muscular dystrophy.

Key words: Confocal microscopy, Duchenne muscular dystrophy, *mdx*, neuromuscular junction

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive, progressive muscle-wasting disease that affects primarily skeletal and cardiac muscle. DMD was first reported in 1868 by Dr. Duchenne de Boulogne, in France, who described the condition as a pseudo-hypertrophic muscular paralysis [23]. Approximately one in 3500 males born worldwide is affected by DMD [24], which is the most common lethal genetic disorder in children [7].

Typically, DMD patients are clinically normal at birth, but with elevated serum levels of the muscle isoform of creatine kinase as a consequence of muscle degeneration. By the age of 2-5 years, the initial physical signs of the disease, characterized by an awkward gait and difficulty in running and climbing stairs, begin to appear, with subsequent hypertrophy of the calf muscles and weakness of the proximal limb muscle. Progressive muscle wasting continues throughout life, initially with weakness of the proximal limb muscles followed by a decrease in lower limb muscle strength and loss of ambulation by about 12 years of age. In the later stages, almost all skeletal muscles are severely involved and the overall clinical course is relentless. Despite supportive procedures, such as long-term mechanical ventilation that can improve the quality of life of the patients, death usually occurs in the early twenties, as a result of cardiac and/or respiratory failures [24].

Several other muscular dystrophies have been described, based on their genetic origin and clinical phenotype (for a review see 8). One of these is Becker muscular dystrophy (BMD), an allelic disorder with a similar, but milder course than DMD, a later age of onset, and a slower rate of progression. There is a clinical continuum between mildly affected BMD patients and severely affected DMD patients, with more than 90% of BMD patients surviving into their 20s and some remaining mobile until old age.

At the cellular level, DMD and BMD involve the loss of skeletal muscle fibers, with marked degeneration. The regenerative capacity of the muscle is usually lost and muscle fibers are gradually replaced by adipose and fibrous connective tissue, which explains the clinical appearance of pseudohypertrophy followed by atrophy [24].

DYSTROPHIN AND THE DYSTROPHIN-ASSOCIATED PROTEIN COMPLEX

The identification of the DMD locus by Kunkel and colleagues about 20 years ago resulted in major advances in the understanding of DMD [40]. DMD and BMD are caused by mutations in the gene encoding dystrophin that result in the absence of dystrophin or in the expression of mutant forms of this protein [33]. The human DMD locus at Xp21 spans about 2.5 Mb and is the largest identified gene, occupying almost 2% of the X chromosome [16].

Correspondence to: Dr. Maria Julia Marques
Departamento de Anatomia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, CEP: 13083-970, Campinas, SP, Brasil. Tel: (55) (19) 3788-6395, Fax: (55) (19) 3289-3124, E-mail: marques@unicamp.br

The DMD gene consists of 79 exons and the corresponding dystrophin transcript is expressed predominantly in smooth, cardiac and skeletal muscle, and in the central nervous system [13,44]. In normal skeletal muscle, the gene produces a 427-kDa dystrophin that is inserted in the cytoplasmic surface of the sarcolemma and is enriched at the myotendinous junctions and the postsynaptic membrane of neuromuscular junctions [62,67,71]. Mutations in the gene result in the absence of dystrophin in the sarcolemma of muscles from DMD patients and in various animal models, including mutant *mdx* mice.

The mdx mice

Mdx mice show a marked deficiency in dystrophin [12] and are the preferred animal model of DMD because the wide availability and low breeding costs. In *mdx* mice, muscle degeneration starts around the third week of age and continues for about one month, with muscle loss being compensated by cycles of regeneration [68]. *Mdx* muscle shows a progressive failure of regeneration in many limb muscles, towards the second year of life [43,56], when the pathology resembles DMD in human patients. In *mdx* mice subjected to excessive cycles of degeneration and regeneration, the exhaustion of myogenic satellite cells, rather than a progressive increase in muscle interstitial fibrosis, could explain the decay in muscle regeneration [36,46]. Skeletal muscles contain several functionally distinct populations of satellite cells and the population that can be evoked by extreme conditions of muscle damage is markedly diminished in *mdx* mice [32].

Despite the differences between the *mdx* phenotype and humans affected with DMD, experiments in *mdx* mice have provided invaluable information about the importance of dystrophin and its protein complex in the pathogenesis of this disease.

Dystrophin

Dystrophin is a member of the spectrin superfamily of proteins [20] and is organized into four structural domains: the amino-terminal actin-binding domain, a central rod domain, a cysteine-rich domain and a carboxy-terminal domain [16,39]. Analysis of deletions in the dystrophin gene of DMD and BMD patients has shown that the amino-terminal actin-binding domain, the cysteine-rich and carboxy-terminal domains are essential for dystrophin function, while the rod domain, which accounts for most of the dystrophin protein [39], can accommodate large, in-frame deletions [55].

The precise function of dystrophin remains unknown. Originally, dystrophin was related to muscle membrane stability, with the lack of dystrophin causing membrane destabilization and increased calcium entry into the muscle fiber, leading to myonecrosis. Indeed, lack of dystrophin is ultimately associated with increased levels of calcium in the muscle fiber and consequent muscle degeneration [6].

The dystrophin-glycoprotein complex (DGC)

The identification of a complex of dystrophin-associated proteins provided important evidence that the dystrophin complex has a role in signaling, in addition to its function in stabilizing the plasma membrane.

The dystrophin-glycoprotein complex (DGC) is a group of protein complexes that includes cytoskeletal actin, the dystroglycan integral membrane proteins, the syntrophins, dystrobrevins and sarcoglycans (Figure 1; for a review see 7,42,58). Mutations in many components of this complex cause other forms of autosomally inherited muscular dystrophy [8]. In DMD patients, the DGC components are dramatically reduced or absent in the sarcolemma compared to normal muscles [26], suggesting that dystrophin is essential for the correct formation of the DGC.

In the DGC model represented in Figure 1, alpha-dystroglycan, an extracellular component of the DGC, is linked to the sarcolemma by interaction with a transmembrane complex consisting of the muscle-specific beta-dystroglycan and sarcoglycan complex [35]. The cytoplasmic tail of beta-dystroglycan binds dystrophin via a WW domain in the cysteine-rich dystrophin regions, and alpha-dystroglycan binds to components of the extracellular matrix, in particular the laminins and agrin, to provide a link between the internal cytoskeleton and the extracellular matrix [25]. The N-terminal regions of dystrophin associate with cytoskeletal actin, although the central rod region of dystrophin also has actin-binding properties. Finally, the C-terminal domain interacts with dystrobrevin and the syntrophins.

The signal transduction cascades associated with the DGC play important roles in cellular defense mechanisms and in the regulation of cell survival and cell death, and suggested that the disruption of specific signaling pathways could contribute to the dystrophic phenotype in DMD patients [58]. The cellular signaling molecules associated with the DGC include calmodulin, Grb2 and nitric oxide synthase (NOS).

Calmodulin

Calmodulin binding sites have been described for both dystrophin and synthrophins [37,53]. Calmodulin-regulated activities are reduced in dystrophin-deficient muscle [54] and the dystrophin-actin interaction is regulated by calmodulin [37]. Although many protein kinases, including calmodulin-dependent kinases, participate in cellular processes that regulate apoptosis [28], their role in calcium handling may be more important and could contribute to the abnormal influx of calcium in dystrophic muscle. In addition to calmodulin, several other molecules involved in calcium homeostasis have recently been investigated in these muscles [19,29,50,60]. Curiously, the clinical and pathological sparing of extraocular muscles may partly reflect their better ability to handle increased levels of calcium [38], and the development of calcium channel blockers for the treatment of DMD is an expanding area of research [8].

Grb2

Beta-dystroglycan binds to Grb2 and SH2/SH3 adapter molecules involved in several intracellular pathways, such as integrin-mediated cell survival [31,66]. In addition, the association of dystroglycan with Grb2 at the neuromuscular junction supports the idea that this linkage is involved in membrane depolarization and neurotransmitter receptor activation [17].

Neuronal nitric oxide synthase (nNOS)

The enzyme nNOS is associated with the DGC through multiple binding sites [1], one of these being syntrophin [9]. nNOS activity is dramatically reduced in the sarcolemma of dystrophin-deficient muscle [10,14]. This association suggested the involvement of nNOS in the pathogenesis of muscular dystrophy, although nNOS null mice do not have a dystrophic phenotype, nor do alpha1-syntrophin deficient mice, which also lack sarcolemmal nNOS [15].

In skeletal muscle, a specific isoform of NOS, NOS-Iμ, produces NO, that is involved in many intracellular signaling pathways. NO-mediated cGMP production regulates several physiological responses and has important signaling actions in inflammation and in the regulation of vascular muscle tone. When muscle nNOS activity is reduced, as in DMD, sympathetic-mediated vasoconstriction is unopposed by NO-mediated vasodilation, resulting in functional ischemia in the muscle [63]. NO also activates satellite cells, the precursor cells of skeletal muscle, and has an important impact on skeletal muscle repair after injury [3]. Together, these findings show that, although a deficiency in NOS is insufficient in itself to produce dystrophy, other aspects of muscle degeneration/regeneration, such as the inflammatory response, vascularization and satellite cell activation are regulated by nNOS activity, which in turn may depend on a normal DGC.

DYSTROPHIN AND THE NEUROMUSCULAR JUNCTION

Much of the work focusing on the importance of dystrophin in synapse structure and function has been based on the neuromuscular junction of dystrophin-deficient *mdx* mice. The neuromuscular junction consists of portions of three cell types, the Schwann cell, motor neuron and muscle fiber, with the synaptic portions of the latter cells being highly specialized for neurotransmission [for a review see 64].

Nerve terminal

The motor nerve terminal is specialized for neurotransmitter release, with a large number of synaptic vesicles that contain acetylcholine (ACh), as well as mitochondria and dense core vesicles that contain calcitonin gene related peptide (CGRP), which has been implicated in acetylcholine receptor (AChR) synthesis and function [11].

During normal development, in the first two weeks after birth, excess nerve terminals are removed by synapse elimination in which the neuromuscular synapse switches from multiple to single innervation [4]. Developmental studies of the dystrophic junction have shown that, at this time, nerve terminals viewed using confocal immunofluorescent techniques are not different from those of control mice, although the dystrophic neuromuscular junction becomes monoinnervated earlier than control junctions [51]. This pattern of monoinnervation could be the result of the increased calcium influx normally seen in dystrophic muscle, or because of mechanical instability between nerve terminals and the postsynaptic membrane or basal lamina components or, because of poor terminal sprouting in the early phases of development. Whatever the mechanisms involved, these results suggest that dystrophin or a normal cytoskeletal complex is important for establishing the innervation pattern of neuromuscular junctions during development.

Nerve terminals in adult dystrophic junctions have a more complex organization than in normal mice, with thin profiles and bulbous enlargements at their

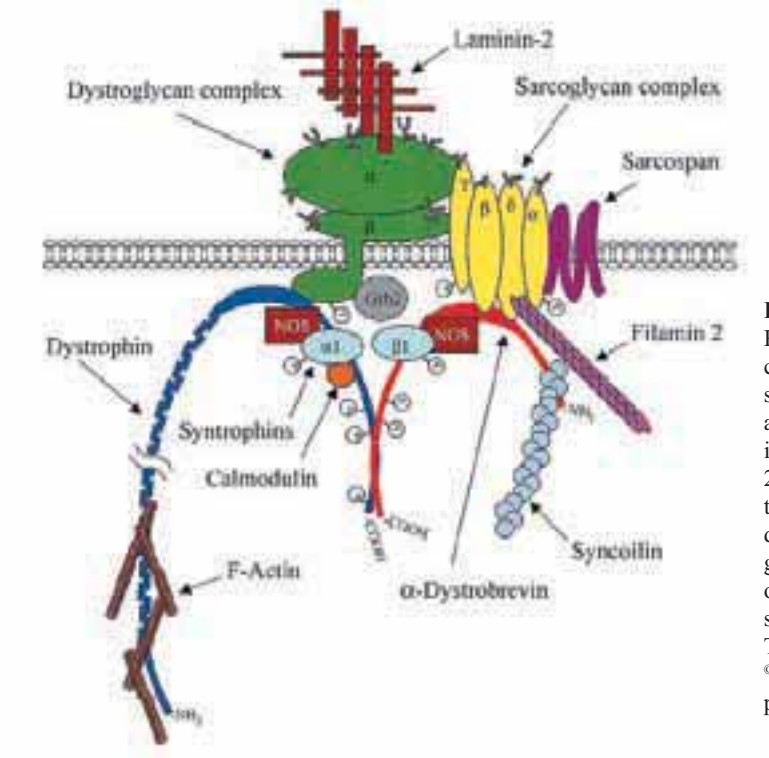

Figure 1. Dystrophin-glycoprotein complex. Representation of the integral components of the complex, dystrophin, the dystrogycan complex, the sarcoglycan complex, α-dystrobrevin, the syntrophins and sarcospan. The extracellular ligand, laminin-2 and intracellular binding sites, F-actin, syncoilin, filamin 2, are shown. The signaling molecules associated with the complex are calmodulin, Grb2, and nNOS. The dystroglycans and sarcoglycans are shown to be glycosylated and phosphorylation sites are represented on dystrophin, α-dystrobrevin, β-dystroglycan, the syntrophins, and α- and γ-sarcoglycans. (From Rando TA. Muscle & Nerve 2001; 24:1575-1594; Copyright © (2001, John Wiley & Sons, Inc. Reprinted with permission of John Wiley & Sons, Inc.).

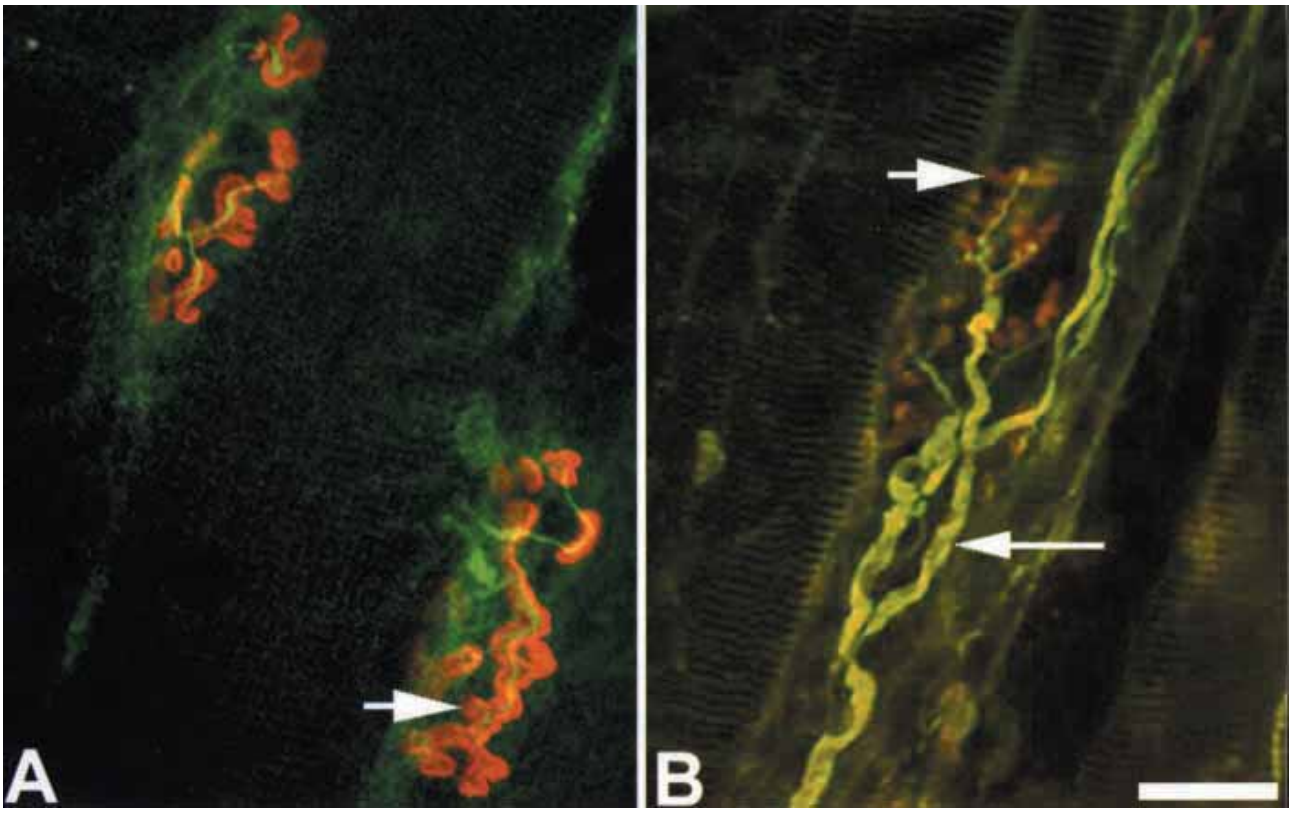

Figure 2. Neuromuscular junction: acetylcholine receptors (**red**) and nerve terminal (**green**) distribution, viewed with fluorescence confocal microscopy. In controls (**A**), AChRs are distributed in continuous branches (**arrow**), covered by the processes of nerve terminal. In *mdx* mice (**B**), AChRs are distributed in small spots (**short arrow**) and nerve terminals show fine arborizations with bulbs, that cover the center of AChRs spots. Intramuscular nerve branches are seen in B (**long arrow**). AChRs labeled with rhodamine-α-bungarotoxin and nerve terminal labeled with anti-neurofilament-FITC. Bars: A = 20 μm, B = 30 μm.

tips [47,65] (Figure 2), and can sprout in response to a nerve lesion to a same extent as a normal muscle. Dystrophic terminals display numerous intraterminal sprouts, possibly in response to sprout-inducing signals from regenerating muscle fibers.

Mdx mice also show changes in the distribution of CGRP [48], which is produced in the cell bodies of motor axons and is transported down the axon to be stored in dense-cored vesicles in the nerve terminal [61]. The changes in CGRP distribution seen in dystrophic muscles correlate with the alterations in the nerve terminal architecture of these mice. The finding that fewer dystrophic junctions are positive for CGRP raises questions as to how CGRP expression is controlled at the neuromuscular junction of dystrophin-deficient fibers in *mdx* mice. One possible explanation is that muscle trophic factors that act on the motor neuron to induce CGRP production [61] are downregulated in dystrophic muscle, thus leading to a decrease in CGRP delivery to dystrophic junctions. Another possibility is that the CGRP receptor complex may be affected by the absence of dystrophin and/or its binding to heparan sulfate [30,45] in the synaptic basal lamina. Considering the importance of CGRP to synaptic remodeling and formation, an assessment of the content of this peptide in dystrophic motoneurons could be important for improving cell-mediated therapies.

Terminal Schwann cells

Terminal Schwann cells are important for maintaining the structural and functional properties of nerve terminals. These cells respond vigorously to nerve damage [59], possibly in order to improve nerve regeneration and muscle reinnervation [34]. Terminal Schwann cells may regulate synaptic function by modulating the production of NO that could act as a local and/or retrograde second messenger [22]. In *mdx* mice, nNOS is dramatically reduced in the sarcolemma of dystrophic muscles [10,14]. nNOS is also dramatically reduced in the presynaptic region of dystrophin-deficient fibers of *mdx* mice, and confocal fluorescence microscopy suggests that this nNOS is associated with terminal Schwann cells [57]. The extent to which the presynaptic region of the *mdx* neuromuscular junction is "dystrophin deficient" is unclear. Dystrophin and C-terminal isoforms of dystrophin are expressed in Schwann cells of the sciatic nerve [27] and superior cervical ganglion [21], but this expression is not affected in *mdx* mice, at least in the superior cervical ganglion. It would be interesting to verify whether dystrophin and its complex are also altered in terminal Schwann cells of *mdx* mice, and how this alteration can contribute to changes in NO production by Schwann cells and their response to muscle disease.

The post-synaptic membrane – Acetylcholine receptors

In normal mice, the postsynaptic membrane is depressed into a gutter beneath the nerve terminal (the primary synaptic cleft) and then further invaginated into junctional folds. These folds result partly from mechanical adhesive forces between the nerve terminal and the postsynaptic membrane [49]. Ultrastructural studies of dystrophic junctions have shown that these folds are sparse and that the primary synaptic cleft is shallow [69]. Junctional folds have a heterogenous molecular organization with different domains. The top of the folds has a high concentration of acetylcholine receptors (AChRs; up to 10,000/μm^2). Cholinergic receptors colocalize with rapsyn, utrophin and α-dystrobrevin-2. Dystrophin, α-dystrobrevin-2 and syntrophin-α1, β1 are localized in the depths of the junctional folds, together with Na^+ channels and N-CAM (for review see 5,64). This elaborate and heterogeneous cytoskeleton serves to generate and maintain the high synaptic density of AChRs, by modulating receptor stability, turnover rate and pattern of distribution.

In *mdx* mice, the lack of a normal DGC would be expected to lead to changes in the molecular organization of AChRs. In *mdx* mice, receptor density is maintained at relatively normal levels in adult muscles, but their rate of degradation is significantly faster than in controls [70]. However, this phenomenon is not related to the absence of dystrophin, since the AChRs of *mdx* mice can be stabilized by elevations in the cAMP levels through the activation of guanylate cyclase [70]. In support of this is the finding that knockout mice for the dystrophin-glycoprotein complex show no changes in receptor stabilization [2]. AChR stabilization in *mdx* muscles may be mediated by neural trophic factors, such as CGRP. This agrees with recent findings that fewer dystrophic junctions are positive for CGRP, when compared to normal junctions. However, a lack of CGRP may not explain the changes in the pattern of receptor distribution seen at the dystrophic neuromuscular junction [48].

The pattern of AChR distribution is dramatically affected in adult dystrophin-deficient muscle fibers. AChRs labeled with rhodamine-α-bungarotoxin show an island-pattern distribution, in contrast to the normal pretzel-pattern of control mice [47,51] (Figure 2). However, since *mdx* mice show cycles of muscle

regeneration, this raises the question of whether the changes in receptor distribution are a consequence of muscle fiber regeneration rather than the absence of dystrophin [47]. Studies of normal regenerated muscle fibers, after injection of the local anesthetic lidocaine, have shown that AChRs break apart into islands, in a pattern of distribution similar to that of dystrophin-deficient muscle fibers [52]. This suggests that neither dystrophin nor an intact DGC are directly involved in AChR distribution. Developmental studies of the dystrophic junction have been useful in showing whether this situation applies to dystrophin-deficient regenerated muscle fibers

During normal development, in the first two weeks after birth, the AChR distribution changes from an oval plaque to a branched form [4]. At dystrophic neuromuscular junctions, the developmental changes in AChRs proceeds normally, i.e., from a plaque to pretzel distribution in a manner similar to control muscles. Studies *in vitro* have also shown that dystrophin is not required for the initial formation of AChR aggregates [41]. Utrophin, which is concentrated at the neuromuscular junction, can compensate for the lack of dystrophin [18], and this could explain the normal pattern of AChR distribution in the *mdx* muscle during development.

Curiously, changes in the AChRs of dystrophic *mdx* mice are only seen at postnatal day 21 [51], which coincides with the appearance of the first regenerated muscle fibers. These findings agree with the previous suggestion that changes in AChR distribution are related to muscle regeneration and that dystrophin or a normal DGC is not involved in alterations in receptor distribution, at least during development.

In conclusion, the lack of dystrophin or a normal DGC may not totally explain the alterations seen in the dystrophic junction. In the case of *mdx* mice, muscle regeneration can partly dictate some of the changes observed. The widespread effects of the lack of dystrophin, such as in the production or action of second messengers, or neuropeptides, may be relevant to a better understanding of the mechanisms involved in neuromuscular synapse formation and maintenance.

CONCLUSION

The knowledge of the complexity of the molecular organization of the dystrophic fiber, with DGC serving as a scaffold for signaling molecules, in addition to its mechanical function in stabilizing muscle membrane, is of great importance to the development of new strategies to treat dystrophinopaties. At the neuromuscular junction, the DGC is necessary for maturation and stabilization of the neuromuscular apparatus, although some structural changes of the dystrophic junction seem to be a consequence of muscle fiber regeneration, rather than to deficiency of dystrophin. Next, it would be important to know how the signaling events associated to the dystrophin complex affect the neuromuscular junction. This may provide information on the role of the dystrophin-complex in the mechanisms regulating the normal regression of synapses during development, as well as pathogenically-induced degeneration of neuromuscular and other synapses.

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