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Review

Recent insights into neuromuscular junction biology in Duchenne muscular dystrophy: Impacts, challenges, and opportunities

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A B S T R A C T

Duchenne muscular dystrophy (DMD) is the most common and relentless form of muscular dystrophy. The pleiotropic effects of dystrophin deficiency include remarkable impacts on neuromuscular junction (NMJ) structure and function. Some of these alterations contribute to the severe muscle wasting and weakness that distinguish DMD, while others attempt to compensate for them. Experimental approaches that correct NMJ biology in pre-clinical models of DMD attenuate disease progression and improve functional outcomes, which suggests that targeting the NMJ may be an effective therapeutic strategy for DMD patients. The objectives of this review are to 1) survey the distinctions in NMJ structure, function, and gene expression in the dystrophic context as compared to the healthy condition, and 2) summarize the efforts, opportunities and challenges to correct NMJ biology in DMD. This information will expand our basic understanding of neuromuscular biology and may be useful for designing novel NMJ-targeted drug or behavioural strategies to mitigate the dystrophic pathology and other disorders of the neuromuscular system.

1. Introduction

Dystrophin is a highly abundant protein localized to the inner face of the plasma membrane of muscle fibres. The primary function of this molecule is to serve as a link between the sarcolemma and cytoskeletal ɣ−actin, which together provide strong structural support for the myofiber during mechanical stress. The absence of dystrophin protein, due to mutations in its gene, *DMD*, results in the most common and relentless form of muscular dystrophy, Duchenne muscular dystrophy (DMD). It has been known for over 30 years that dystrophic skeletal muscle is characterized by pathophysiological alterations in pre- and postsynaptic neuromuscular junction (NMJ) structure and function, which contribute to the hallmark muscle wasting and weakness in DMD [1−13]. Experimental approaches that impact skeletal muscle and affect NMJ biology in pre-clinical models of DMD attenuate disease progression and improve functional outcomes [14−18]. This evidence suggests that targeting the NMJ may be an effective therapeutic strategy for DMD patients. Thus, continued examination of the mechanisms that govern NMJ morphology and function in DMD will expand our comprehension of the basic biology of the disorder, as well as identify opportunities for innovative treatment approaches. In the current paper, we begin by reviewing recent progress in DMD research, with particular emphasis on novel salutary modalities. We then discuss NMJ biology in the healthy and dystrophic contexts and follow with a detailed analysis of the effects of normalizing the NMJ in muscular dystrophy. In closing, we highlight the most significant remaining knowledge gaps, as well as opportunities for future pursuit that will advance our understanding of, and therapeutic options for, the NMJ in DMD. This article expands on earlier, excellent reviews of similar themes [19−23].

2. Duchenne muscular dystrophy

DMD is the most common congenital neuromuscular disorder affecting approximately 1 in 6000 live male births [24,25]. The economic burden associated with DMD is significant for patients ($23,000−$54,000 USD) and their families ($58,000−$71,000 USD) all over the world [26]. Natural history studies demonstrate that boys with DMD experience progressive proximal muscle weakness and wasting at approximately 2−5 years of age, which is also accompanied by a delay in motor milestone achievements [25,27]. This gradual loss in limb function typically necessitates ambulatory supports by early adolescence. Other clinical hallmarks that manifest in these patients include the accumulation of intramuscular fatty and fibrotic tissue resulting in excessive enlargement (i.e., pseudohypertrophy), particularly of the gastrocnemius muscle, as well as a positive Gowers' sign that presents as a predictable difficulty rising from a lying supine position. Respiratory and/or cardiac failure claim most DMD patients in their third or fourth decades.

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DMD is caused by mutations in the *DMD* gene and the subsequent absence of its dystrophin protein product. Without dystrophin, its eponymous oligomeric structure termed the dystrophin-associated protein complex (DAPC) cannot be formed [27,28]. Highly localized along the sarcolemma, the DAPC is a critical signalling apparatus between the extracellular matrix and intracellular cytoskeleton that serves to maintain the structural integrity of muscle cells. The lack of dystrophin, and by extension the DAPC, ultimately lead to repetitive cycles of degeneration/regeneration, chronic inflammation, and muscle atrophy [25,29,30]. While dystrophin is certainly important, other members of the DAPC also play critical roles in health and disease. Although often overlooked when discussing the devastating effects of DAPC deficiency, NMJ structure and function are remarkably impacted in muscular dystrophies, DMD in particular (Fig. 1) [1−13]. Veritably, the biology of dystrophic NMJs is significantly compromised in several pre-clinical models, which strongly suggests that this phenomenon contributes to the muscle wasting and weakness apparent in DMD patients. This is not surprising considering the critically important role of the NMJ in regulating skeletal muscle phenotype and contractile activity [31].

There is currently no effective treatment for DMD [32]. Symptomatic patients are prescribed glucocorticoids as a standard of care, which attenuates the decline in muscle strength and function while also delaying the onset of catastrophic respiratory and cardiac dysfunction [25,33]. However, long-term corticosteroid usage results in detrimental side effects such as abnormal behaviour and weight gain. Vamorolone (ReveraGen BioPharma) and Edaslonexent (Catabasis Pharmaceuticals), two anti-inflammatory medications currently in the latter phases of clinical trials, provide similar therapeutic effects of glucocorticoids without the adverse off-target effects [33]. Other emerging therapies include exon-skipping compounds, such as Viltepso (NS Pharma), Exondys 51 and Vyondys 53 (Sarepta Therapeutics), as well as the nonsense mutation suppressor, Translarna (PTC Therapeutics), which have been approved by the U.S. Food and Drug Administration and European Medicines Agency, respectively. However, these therapeutic strategies are not without their limitations. For example, Vyondys 53 might be effective only for those who have a *DMD* mutation that is germane to the skipping of exon 53, which represents approximately 8% of all DMD patients [34]. Moreover, premature termination codon readthrough capability could successfully address disease-causing mutations in a maximum of 15% of boys with DMD [35]. A common shortcoming of these genetic therapies is the restoration of a truncated and less functional dystrophin, which will ultimately translate, in a best case scenario, to a milder, Becker-like dystrophic phenotype [36]. Thus, there is a critical, unmet clinical need to identify effective curative approaches that apply to all DMD patients.

3. Development, maintenance and plasticity of the NMJ

The NMJ is an electrochemical signalling apparatus that lies at the interface between an α-motoneuron (αMN) and the skeletal muscle cells that it innervates. More specifically, it is comprised of an αMN nerve terminal, the endplate region of a muscle fibre, and perisynaptic Schwann cells that envelop the synapse [20,22,23,37]. Depolarization of the αMN terminal prompts the presynaptic exocytosis of acetylcholine (ACh)-containing vesicles. ACh from a single vesicle (i.e., quantal content) diffuses into the synaptic cleft and is either destroyed by acetylcholinesterase or binds to ACh receptors (AChR) to evoke a miniature endplate potential (mEPP) at the postsynaptic endplate. The summation of mEPPs elicited from a simultaneous release of multiple ACh transmitter quanta upon a single nerve impulse will cause a relatively larger, local postsynaptic depolarization known as an endplate potential (EPP). In the healthy condition, the EPP is almost always sufficient to surpass the threshold required for depolarization of the sarcolemma, which then causes a muscle

action potential to ensue and leads to the contraction of all myofibers innervated by the αMN. The amplitude by which the EPP surpasses the depolarization threshold is known as the "safety factor", and disruption to the morphology and/or function of the NMJ will lower this safety factor below depolarization threshold leading to transmission failure, muscle weakness and dysfunction [38].

The development and maturation of the NMJ is well characterized in rodents [22,23,39]. As early as embryonic day (E) 9.5, myofibers develop with broadly dispersed AChRs that subsequently accumulate to the central region of the fibre, a process known as pre-patterning. Shortly after at E11-12, motor axons are directed towards this central, pre-patterned domain [21]. The axons first branch, then extend to appose these AChRs to form nascent NMJs [22]. These newly formed, oval-like plaques are innervated by multiple nerve terminals that compete with each other in an activity-based manner to seize territory within an AChR cluster. Polyinnervation of AChR clusters is eliminated by approximately 14 days post-birth (P), when a dominant presynaptic terminal has been established [23]. Subsequently, these more mature synapses adopt a perforated, pretzel-like morphology. The maturation and stability of the NMJ is governed by multiple proteins including the canonical agrin/low-density lipoprotein receptor-related protein 4 (LRP4)/muscle-specific kinase (MuSK) signalling axis, downstream targets docking protein 7 (Dok7), and 43 kDa receptor-associated protein of the synapse (rapsyn), as well as additional synaptic gene regulators such as GA-binding protein (GABP; also known as nuclear respiratory factor 2, or NRF-2), and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α). Other proteins, such as utrophin and protein kinase A (PKA) also modulate the morphology and function of the NMJ. While many additional signalling pathways have been identified that play a role in synapse specialization, including neuregulin/ErbB, Wnt/β-catenin, and Hippo/Yes-associated protein signalling cascades [40], the remainder of this section will emphasize molecules that have been examined in the DMD context and how they develop, maintain, and remodel synaptic structure and function. Readers interested in much more comprehensive surveys of NMJ maturation are enthusiastically referred to these recent, expert reviews [20,22,23,37,40,41].

3.1. Agrin/LRP4/MuSK signalling

Agrin was identified as the first neurotrophic factor to regulate NMJ biology [21,22]. The proteoglycan governs the expression and localization of AChRs and is therefore critical for the postnatal development and plasticity of the NMJ. Indeed, although rodents lacking agrin form AChR clusters, they are unstable and exhibit a dispersed organization in the broad middle region of myofibers, resembling an arrangement that is observed during development [22,39,41]. Additionally, recombinant agrin treatment of denervated muscles eliminates ectopic AChR cluster formation, further demonstrating the importance of this neural factor [39,40]. Agrin exerts its effects by initiating the LRP4/MuSK signalling cascade, which is essential for its downstream control of AChR expression. For instance, the agrin/LRP4/MuSK interaction promotes the expression of several synaptic proteins that encourage AChR transcription, stabilization, and turnover. These effector molecules include rapsyn, Dok7, Abl tyrosine kinase, geranylgeranyltransferase, Rho GTPases, and P21-activated kinase 1 [22,39]. While many of these factors exhibit functional redundancy and are therefore dispensable for the postnatal development of the NMJ, Dok7 and rapsyn are essential components of the postsynaptic apparatus [22]. Additional evidence for the importance of the agrin/LRP4/MuSK signalling axis is demonstrated by LRP4 and MuSK knockdown and knockout (KO) studies [20−22]. For example, LRP4 KO mice exhibit perinatal lethality due to severe impairments in NMJ postsynaptic endplate formation [42]. Thus, the agrin/LRP4/MuSK cascade serves as the regulatory centrepiece for the maturation and remodelling of the NMJ.
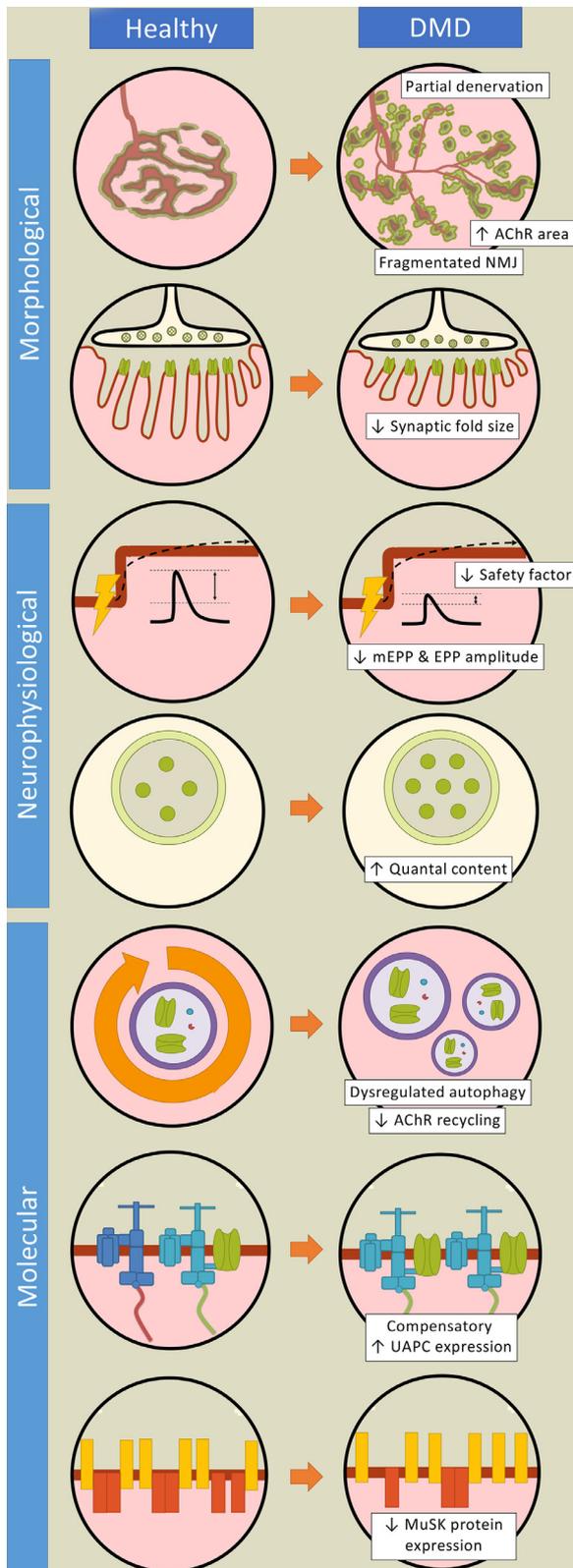
Fig. 1. Neuromuscular junction biology in Duchenne muscular dystrophy. Evidence from Duchenne muscular dystrophy (DMD) patients and pre-clinical studies demonstrate numerous differences in neuromuscular junction (NMJ) structure, function, and gene expression between the healthy and dystrophic conditions. Morphological abnormalities of the synapse exhibited in DMD include denervation, fragmentation and denuded postsynaptic folds. Acetylcholine receptor (AChR) expression is also impacted. Neurophysiological properties such as impulse transmission also differ in dystrophic muscle. For instance, reduced endplate potential (EPP) and miniature EPP (mEPP) amplitudes, as well as a depressed safety factor, compromise the reliability of neurotransmission in dystrophic muscle. Furthermore, several disparities in the

3.2. Transcriptional regulators of synaptic genes

The links between agrin/LRP4/MuSK and synaptic gene expression are still being identified. Established players include transcription factors that are part of the E26 transformation-specific (Ets) family of proteins, including GABPα/β and Ets variant 5 (Erm) [20]. These factors are also regulated at the synapse by mitogen activated protein kinase pathways, including c-Jun N-terminal kinases and extracellular signal-regulated kinase (ERK) signalling [40]. An abundance of subsynaptic genes contain a common, conserved sequence in their promoters known as the N-box motif (CCGGAA). Here, in subsynaptic myonuclei (also known as fundamental myonuclei), Ets transcription factors bind and facilitate the transcription of synaptic genes, including AChR subunits (*Chrnδ and Chrnε*), acetylcholinesterase (*AChEst*), *Musk, Rapsn*, and utrophin (*Utrn*). Although Ets factors are dispensable for synapse formation [43−45], these proteins play a remarkable role in NMJ remodelling. For example, rodents lacking GABPα/β exhibit impaired postsynaptic development as a result of attenuated agrin signalling and reduced subsynaptic gene transcription [43,44]. Furthermore, Hippenmeyer and colleagues demonstrate severe impairments in NMJ plasticity and function in animals lacking Erm [45]. This evidence underscores the importance of Ets transcription factors, specifically GABPα/β and Erm, in regulating the NMJ gene expression program and synaptic function.

The transcriptional coactivator PGC-1α is another important modulator of the NMJ [28,46,47]. PGC-1α is regulated through several post-translational modifications that are mediated by upstream molecules such as adenosine monophosphate-activated protein kinase (AMPK), which is critical protein that governs neuromuscular system biology [28]. Mechanistic links have been elegantly established between PGC-1α and synaptic gene regulation via GABPα/β/N-box signalling [15,48]. Further evidence for the role of PGC-1α in the NMJ gene program can be observed *in vivo* [17,18,49,50]. For instance, in addition to demonstrating slower, and more oxidative muscle characteristics such as mitochondrial biogenesis and type 1 and 2a myosin heavy chain expression, mice overexpressing PGC-1α specifically in skeletal muscle exhibit a strong postsynaptic gene expression signature, as well as a type 1 αMN phenotype [50,51]. Interestingly, the latter alteration indicates that the transcriptional coactivator mediates a retrograde signalling network from myofibers to their innervating αMN. Elaborating on this phenomenon, Mills and colleagues recently showed in cell culture experiments that a PGC-1α isoform directs axon recruitment and NMJ formation through the release of the myokine, neurturin [52]. Thus, interventions that stimulate PGC-1α in skeletal muscle, like exercise for example, may evoke adaptive plasticity throughout the peripheral neuromuscular system. This warrants further research into the therapeutic potential of this protein.

PGC-1α-mediated induction of utrophin via GABPα/β/N-box signalling has been demonstrated in numerous studies [15,48,53]. Utrophin is the key component of the utrophin-associated protein complex (UAPC), an oligomeric structure that is homologous to the DAPC. However, unlike the DAPC, which resides in the troughs of the postsynaptic folds at the NMJ and along the length of the sarcolemma, the UAPC is typically concentrated at the crests of the postsynaptic invaginations, but can also be found to a lesser extent extrasynaptically [54]. In fact, slow-twitch, oxidative muscles possess significantly greater amounts of extrasynaptic utrophin compared to faster, more glycolytic muscles, which is thought to be due, in part, to

molecular biology of the NMJ between the healthy condition and DMD have been documented and include dysregulated autophagy, altered cyclic adenosine monophosphate (cAMP) signalling, as well as attenuated muscle specific kinase (MuSK) expression. Notably, a significant upregulation of synaptic and extrasynaptic utrophin content, as well as the larger utrophin-associated protein complex (UAPC), are hallmark characteristics of dystrophic muscle and are believed to compensate functionally for the lack of dystrophin.

enhanced transcriptional and post-transcriptional processes in slow, oxidative myofibers [53]. Nonetheless, work from Deconinck et al. and Grady et al. demonstrated that the absence of utrophin only modestly affects NMJ biology [55,56]. The true impact of utrophin is revealed in the context of DMD, which will be discussed in greater detail later in our survey.

Another notable target of PGC-1α and GABPα/β/N-box transcriptional activation are the *Chrn* genes, including the δ and ε subunits [15,20,40]. The density of synaptic AChRs is regulated by processes that synthesize, stabilize, and dismantle the receptors. Innervated AChRs typically possess a half-life of ~14 days, after which they are endocytosed from the motor endplate and either stored in intracellular compartments, recycled back to the synapse, or degraded [19]. The fate of AChRs is heavily influenced by their phosphorylation status and stability, which are governed by the cAMP/protein kinase A (PKA) signalling pathway [19,37]. AChR elimination involves autophagic machinery such as autophagy related 7 (ATG7), muscle ring finger 1 (MuRF1), sequestosome 1, E3 ubiquitin-protein ligase TRIM63, and Rab5-GTPase [19,57]. Mechanisms that regulate AChR biology pose as a potential therapy for DMD and will be further discussed below.

4. Synaptic biology in DMD and therapies targeting the NMJ

Early studies in DMD patients and *mdx* mice revealed that the absence of dystrophin resulted in fragmented NMJs and denuded postsynaptic folds [4,6,7,12,13,58], as well as an accelerated degradation of AChRs [59] (Table 1). These alterations, which are also observed in ageing NMJs, may not necessarily translate into functional impairments in neuromuscular transmission [60], whereas a simplified endplate morphology can contribute to the loss of crucial postsynaptic membrane proteins [61]. These initial observations in postsynaptic alterations have since been reported numerous times in *mdx* mice [9–11,62–64], golden retriever muscular dystrophy dogs [65], and other animal models of DMD [66,67]. Whether these changes occur independent from dystrophy-induced muscle degeneration/regeneration is debated [68]. The absence of dystrophin also alters the presynaptic apparatus of the NMJ likely due, in part, to fragmentation of the motor endplate [11]. Specifically, an increase in presynaptic nerve terminal branching, as well as partial denervation have been observed in *mdx* animals [11], while axonal sprouting occurs in DMD patients and mice [13,69].

Initial electrophysiology experiments revealed modest declines in mEPP and EPP amplitudes and a compensatory increase in quantal content in the diaphragm of *mdx* mice, which is the muscle that most closely phenocopies the human dystrophic pathology (Table 2) [6,7]. More recently, Van der Pijl et al. demonstrated augmented quantal content, and reductions in mEPP amplitude and the safety factor for neurotransmission in *mdx* mice, as well as in the more severe dystrophin-utrophin double knockout animals [66]. Additionally, high-strain, low-repetition, eccentric muscle damage in *mdx* mice results in a loss of force production, as well as exacerbates pre-existing impairments in neuromuscular transmission and fragments the

Table 1
NMJ morphology in DMD NMJ structure in pre-clinical models of DMD versus healthy littermates.

Reference	DMD model	Age	Muscles studied	Presynapse	Postsynapse
Torres and Duchen 1987	*mdx*	4 wk 7 wk 4 mo	Soleus	Not reported	Reduced synaptic fold size (all age groups) Fragmented (4 mo)
Nagel *et al.*, 1990	*mdx*	2.5 wk 8.5 wk 37 wk	Diaphragm	Not reported	Reduced synaptic fold size (all age groups) Increased AChR area (2.5 wk, 37 wk) Similar AChR area (8.5 wk)
Lyons and Slater 1991	*mdx*	8 wk	EPT	Not reported	Reduced synaptic fold size Fragmented
Grady *et al.*, 1997	*mdx* *mdx:utrn*$^{-/-}$	8 wk 8 wk	Sternomastoid Sternomastoid	Not reported Not reported	Reduced synaptic fold size Reduced synaptic folds size
Santa Neto *et al.*, 2003	*mdx*	5–6 mo	Sternomastoid	Axon sprouting	Fragmented
Minatel *et al.*, 2003	*mdx*	P1 1 wk 2 wk 3 wk	Sternomastoid	Greater monoinnervation (P1, 1 wk) Similar innervation (2 wk, 3 wk)	Similar morphology (P1, 1 wk, 2 wk) Fragmented (3 wk)
Personius and Sawyer 2006	*mdx*	6–8 mo	Diaphragm	Not reported	Fragmented
Marques *et al.*, 2008	*mdx*	1 mo 6 mo	Sternomastoid	Not reported	Fragmented (both age groups)
Ferretti *et al.*, 2011	*mdx*	2 mo	Intrinsic laryngeal, sternomastoid	Not reported	Fragmented
Pratt *et al.*, 2013	*mdx*	2-3 mo	Quadriceps	Not reported	Fragmented (worsened with damage)
Pratt *et al.*, 2014	*mdx*	3 mo	Quadriceps	Not reported	Fragmented (worsened with damage)
Pratt *et al.*, 2015	*mdx*	3 mo	Quadriceps	Increased nerve branching Partial innervation	Fragmented (worsened with damage)
Van der pijl *et al.*, 2016	*mdx* *mdx:utrn*$^{-/-}$	2–6 mo 1–2 mo	EPT, diaphragm EPT, diaphragm	Not reported Not reported	Fragmented Fragmented
Van der pijl *et al.*, 2018	*mdx* *mdx-XistΔhs*	2–5 mo 2–5 mo	EPT, diaphragm EPT, diaphragm	Not reported Not reported	Fragmented Fragmented
Haddix *et al.*, 2018	*mdx*	P38 P66 P450	Sternomastoid	Similar nerve branching	Fragmented (all age groups) Fragmentation (worsened with age)
	GRMD	1–6 yr	Cranial tibial	Similar nerve branching	Fragmented Increased AChR area
NMJ structure in DMD patients compared to healthy participants					
Reference	*Cohort size*	*Age*	*Muscles studied*	*Presynapse*	*Postsynapse*
Jerusalem 1974	3	3–6 yr	Peroneus brevis	Similar terminal size Similar vesicle size	Reduced synaptic fold size Similar AChR area
Harriman 1976	13	4–8 yr	Vastus internus, gastrocnemius, deltoid, palmaris longus, peroneus brevis	Increased axonal sprouting Partial innervation	Reduced synaptic fold size
Sakakibara et al., 1977	3	5–11 yr	Intercostal	Not reported	Reduced synaptic fold size Similar AChR area

DMD, Duchenne muscular dystrophy; AChR, acetylcholine receptor; EPT, epitrochleoanconeus; GRMD, golden retriever muscular dystrophy dog; NMJ, neuromuscular junction; wk weeks; mo, months; yr, years; P, postnatal day.

Table 2
NMJ electrophysiology in DMD Electrophysiology in pre-clinical models of DMD versus healthy littermates.

Reference	DMD model	Age	Muscles studied	Electrophysiological characteristics
Nagel *et al.*, 1990	*mdx*	2.5 wk 8.5 wk 37 wk	Diaphragm	Similar quantal content (2.5 wk) Increased quantal content (8.5 wk, 37 wk) Similar mEPP amplitude (2.5 wk, 8.5 wk) Reduced mEPP amplitude (37 wk)
Lyons and Slater 1991	*mdx*	8 wk	EPT	Similar quantal content Similar mEPP amplitude Similar mEPC amplitude Similar resting postsynaptic membrane potential
Grady *et al.*, 1997	*mdx*	8 wk	Sternomastoid	Similar mEPP amplitude Similar neuromuscular transmission fatigue
	mdx:utrn$^{-/-}$	8 wk	Sternomastoid	Similar mEPP amplitude Similar neuromuscular transmission fatigue Reduced twitch contraction force Increased twitch relaxation time
Deconinck *et al.*, 1997	*utrn*$^{-/-}$	8 wk	EDL and diaphragm	Similar EPP amplitude Similar EPC amplitude and delay time Reduced mEPP amplitude Similar mEPP frequency Similar quantal content
Carlson and Roshek 2001	*mdx*	5−7 wk 6−24 mo	Diaphragm	Reduced mEPP amplitude (both age groups) Similar mEPP amplitude variance (5-7 wk) Increased mEPP amplitude variance (6-24 mo)
Personius and Sawyer 2006	*mdx*	6−8 mo	Diaphragm	Similar neuromuscular transmission fatigue Reduced peak twitch force Increased twitch relaxation time Increased twitch contraction force variability
Pratt *et al.*, 2013	*mdx*	2−3 mo	Quadriceps	Increased neuromuscular transmission fatigue Reduced CMAP amplitude Reduced isometric force production
Pratt *et al.*, 2014	*mdx*	3 mo	Quadriceps	Increased neuromuscular transmission fatigue Reduced isometric force production
Van der pijl *et al.*, 2016	*mdx*	2−6 mo	Diaphragm (electrophysiology, force kinetics) EPT, and GSP complex (EMG)	Increased quantal content Reduced mEPP amplitude Reduced safety factor Increased neuromuscular transmission fatigue Reduced CMAP amplitude Reduced tetanic and twitch contraction force
	mdx:utrn$^{-/-}$	1−2 mo	Diaphragm (electrophysiology, force kinetics) EPT, and GSP complex (EMG)	Increased quantal content Reduced mEPP amplitude Reduced safety factor Increased neuromuscular transmission fatigue Reduced CMAP amplitude Reduced tetanic and twitch contraction force
Van der pijl *et al.*, 2018	*mdx*	2−5 mo	Diaphragm (electrophysiology, force kinetics, IHC) EPT, and GSP complex (EMG)	Increased quantal content Increased EPP rise time, half width, and decay time Reduced EPP and mEPP amplitude Similar mEPP frequency Increased neuromuscular transmission fatigue Reduced CMAP amplitude
	mdx-Xist∆hs	2−5 mo	Diaphragm (electrophysiology, force kinetics, IHC) EPT, and GSP complex (EMG)	Increased quantal content Increased EPP rise time, half width, and decay time Reduced EPP and mEPP amplitude Similar mEPP frequency Increased neuromuscular transmission fatigue Reduced CMAP amplitude

Electrophysiology in DMD patients compared to healthy participants				
Reference	*Cohort Size*	*Age*	*Muscles studied*	*Electrophysiological characteristics*
Panayiotopoulos 1974	9	3−12 yr	Extensor digitorum brevis	Reduced motor unit action potential Similar peak terminal latency
Sakakibara 1977	3	5−11 yr	Intercostal	Similar quantal content Similar mEPP amplitude and frequency Reduced resting postsynaptic membrane potential
Hilton-Brown and Stalberg 1983	8	8−19 yr	Extensor digitorum communis	Increased jitter
Sharma et al., 1995	11	5−10 yr	Tibialis anterior	Similar neuromuscular transmission fatigue Reduced CMAP amplitude Reduced tetanic and twitch contraction force production Increased tetanic and twitch relaxation time

CMAP, compound muscle action potential; DMD, Duchenne muscular dystrophy; EMG, electromyography; EPC, endplate potential current; EPP, endplate potential; EPT, epi-trochleoanconeus; GSP, gastrocnemius-soleus-plantaris; mEPC, miniature EPC; mEPP, miniature EPP; hr, hours; mo, months; wk, weeks; yr, years.

postsynaptic apparatus [9−11]. This drop in force may also be attributed to depressed muscle excitability secondary to the loss of sarcolemmal integrity [70−72]. Nonetheless, it is very likely that both neuronal and muscle impairments contribute to reduced muscle activity, and elevated wasting and weakness in the dystrophic condition. Consistent with rodent work, electrophysiological metrics such as impulse transmission, resting membrane potential, and the compound muscle action potential, are also affected in DMD patients

(Table 2) [4,73−75]. Collectively, these data indicate that dystrophin is essential for the proper maturation of the synapse and is required for optimal neurotransmission at the NMJ.

4.1. Agrin/LRP4/MuSK signalling in DMD

The fragmented nature of the synapse in dystrophic muscle indicates that the mechanisms controlling NMJ morphology are dysregulated, specifically those that govern the post-synaptic apparatus. The absence of dystrophin dissociates the DAPC and its key agrin signalling machinery important for NMJ maturation [20,76]. For example, the loss of the DAPC component dystroglycan prevents agrin binding and disorganizes AChR clustering [76,77]. Furthermore, reduced MuSK mRNA and protein expression can be observed in the quadriceps muscles of animals lacking dystrophin [9,10]. Partial inactivation of the kinase, through transgenic ablation or MuSK autoantibodies, accelerates the loss of AChRs and impairs neurotransmission [78−80], which are characteristics of dystrophic synapses. This suggests that MuSK content and/or activity is attenuated in DMD, however firsthand data in patients are currently lacking.

Despite its lower expression and function levels in dystrophic muscle, the agrin/LRP4/MuSK signalling cascade may serve as valid candidates for muscular dystrophy therapies. Agrin-derived constructs, known as mini-agrins, ameliorate neuromuscular transmission and AChR clustering in murine models of congenital myasthenic syndrome (CMS) and limb gridle muscular dystrophy [41], as well as increase utrophin expression in cultured myotubes [81]. The use of agrin-based therapies has not yet been evaluated in the context of DMD. Augmenting MuSK expression in mdx animals via an adeno-associated virus (AAV) vector reduced neuromuscular failure from eccentric muscle damage and elevated DAPC/UAPC components [16]. Furthermore, AAV induction of downstream effector proteins rapsyn and Dok7 conferred unique neuromuscular alterations in several disparate models of NMDs, including DMD [16], CMS [82], SMA [83], and amyotrophic lateral sclerosis [84]. Thus, these pre-clinical data indicate that targeting the agrin/LRP4/MuSK cascade may eventually yield promising therapeutic effects in DMD patients.

4.2. Regulation of the NMJ gene expression program in DMD

Surprisingly, GABPα/β biology has not yet been directly examined in dystrophic muscle. However, we can infer the stimulation of this signalling pathway in DMD since a number of N-box-containing genes are elevated, such as Utrn and Chrn, which will be discussed in greater detail below. An alternative regulator of the N-box, ERK, may also be partly responsible for this selective increase of synaptic genes, as ERK signalling is upregulated in mdx mouse muscle [85]. The potential regulatory role of PGC-1α in GABPα/β/N-box signalling in dystrophic muscle [15,48], as well as the function of the transcriptional coactivator as a master regulator of neuromuscular phenotype [50,51], has led to an interest in PGC-1α as a possible therapeutic target in DMD. Indeed, several studies have shown that augmenting PGC-1α expression via genetic, pharmacologic, and physiological means, can reduce the dystrophic phenotype in pre-clinical models of DMD (see [28,53] for comprehensive review). The mechanisms by which PGC-1α exerts its beneficial effects in DMD include evoking the NMJ gene expression program, as demonstrated by increased Agrn, Musk, and Chrn expression [15,17,18]. Moreover, PGC-1α also drives the expression of slower, more oxidative characteristics, which endows muscle with a greater degree of protection against the dystrophic phenotype. A notable indicator of the slow, oxidative phenotype is high synaptic and extrasynaptic utrophin expression [53,86], however it is unclear whether utrophin is required for improved molecular and physiological outcomes in mdx mice elicited by, or associated with, PGC-1α induction [18,87].

Regardless of whether utrophin is necessary or not for the effects of PGC-1α, the dystrophin homologue is nevertheless a critically important NMJ molecule when considering potential therapeutic approaches for DMD. An endogenously expressed protein, utrophin content is consistently upregulated in the skeletal muscles of pre-clinical DMD animal models, as well as in DMD patients [88]. This is very likely a compensatory adaptation in an effort to account for the absence of dystrophin. In fact, some data indicate that a positive relationship exists in DMD patients between utrophin protein expression in skeletal muscle and the age of symptom onset [89], while other results indicate no correlation [90]. In mdx mice, further induction of utrophin significantly mitigates the dystrophic phenotype, as well as rescues NMJ morphology and improves synaptic function [27,29,53]. Conversely, the absence of utrophin significantly exacerbates the dystrophic pathology in mdx animals and results in a relatively more accurate phenocopy of the human DMD condition [28]. Therefore, utrophin is clearly an important molecule at the NMJ with substantial therapeutic potential in DMD. Successful translation of utrophin-mediated strategies in patients remains a high priority.

In dystrophic muscle, impaired cAMP/PKA signalling destabilizes and expedites the elimination of AChRs [37,59]. However, this loss is coincidentally offset by the upregulation of Chrn gene expression [11,91] and AChR incorporation at the synapse [59], which therefore maintains net AChR content at the motor endplate at a similar level as compared to the healthy, non-dystrophic condition. Interestingly, attenuating the accelerated loss of AChRs through cAMP signalling also decreases the rate of total protein degradation in skeletal muscle [59]. Several pre-clinical studies with mdx animals have demonstrated the therapeutic applicability of β_2 adrenergic receptor agonists, which are potent stimulators of cAMP production and downstream signalling [92]. Specifically, chronic, low doses of β_2 agonists, including formoterol and clenbuterol, reduce muscle degeneration and increase force production in mdx animals [92]. Clinical studies investigating the effects of cAMP agonists are limited in number, but have also demonstrated improvements in muscular health in Duchenne and Becker patients [93,94]. These β_2 agonist-driven benefits are likely caused, at least in part, through cAMP/PKA signalling at the NMJ, and suggest that regulating AChR biology may provide some therapeutic utility for DMD in the future.

4.3. Activity-induced plasticity of the dystrophic NMJ

Synaptic activity is essential for maintaining the structure and function of the NMJ [23]. Increases or decreases in neural stimulation drives remodelling of the neuromuscular synapse [19,95]. For example, advanced ageing is commonly associated with decreased peripheral nerve activity, NMJ fragmentation and neurotransmission impairments, which are all mitigated by chronic exercise [19,46]. The favourable alterations at the NMJ represent only a tiny fraction of the health benefits provided by habitual physical activity. The underlying molecular mechanisms responsible for exercise-induced NMJ adaptations remain undefined, but likely include MuSK upregulation, AMPK and PGC-1α signalling, and AChR stabilization [46]. To our knowledge, the data on exercise and the NMJ in DMD are limited. For instance, daily, volitional physical activity increased utrophin protein expression in mdx mice [96], and chronic, endurance exercise augmented Utrn in DMD patients [97]. In the absence of additional primary data, we can reasonably speculate as to what impacts exercise would have on neuromuscular synaptic morphology and function in DMD. For example, given that autophagy is essential for optimal NMJ function in the healthy condition, including AChR turnover [98], and exercise robustly stimulates autophagic processes in healthy and dystrophic skeletal muscle [99], then exercise-induced autophagy may act to rescue dystrophic NMJ morphology and function. Moreover, since low-intensity exercise preserves muscle health and physical function in DMD patients [100], it is likely that, as the interface

between muscles and their motor nerves, the NMJ is also positively affected. Examining physical activity-evoked alterations in the structure and function of the NMJ in the DMD context, as well as the underlying molecular mechanisms driving phenotypic plasticity, will increase our understanding of the biology of the neuromuscular system and may lead to the identification and development of novel therapeutic strategies for this disorder.

4.4. Restoration of dystrophin

Perhaps the ideal resolution for the neuromuscular defects in DMD, including at the NMJ, is to restore dystrophin. Indeed, in *mdx* mice only 5−15% of normal dystrophin levels is sufficient to augment muscle function and prolong survival [101], whereas a higher requirement, estimated to be 20−50%, is needed to improve synaptic morphology and function [67]. However, translating this solution to the clinic is challenging. This is due, in part, to the exceptionally large size of the *DMD* gene, which limits the viability of some dystrophin-based gene therapies. On the other hand, delivery to the muscles of truncated variants of dystrophin, for example mini- or micro-dystrophin, leads to the reassembly of the DAPC and significantly attenuates the dystrophic pathology [29]. For instance, treatment of *mdx* animals with mini-dystrophin rescued postsynaptic fold complexity [2]. As

small molecule, viral, and other dystrophin-based gene and cell therapies improve in their efficacy, safety and practicality, we anticipate correction of the NMJ and amelioration of the broader dystrophic pathology.

5. Outstanding questions

The accumulation of synaptic alterations and molecular dysregulation are common in pre-clinical models of DMD. Indeed, the absence of dystrophin significantly impacts NMJ structure, function, and gene expression. This is exemplified, in part, by increased NMJ fragmentation, reduced postsynaptic folding, and elevated neuromuscular transmission variability and fatigue that contribute to the progressive muscle atrophy and weakness that characterize pre-clinical DMD, and to a lesser extent due to the paucity of human studies in this area, DMD patients (Fig. 1, Tables 1 and 2). Molecular mechanisms underlying NMJ adaptations have been identified and include alterations in the agrin/LRP4/MuSK, GABPα/β/N-box, PGC-1α, and cAMP/PKA signalling pathways. Importantly, additional work is necessary to further confirm the importance of these cascades in DMD patients. Several pre-clinical, proof-of-principle studies employing genetic or pharmacological approaches have recently demonstrated that targeting these pathways attenuate NMJ morphological and
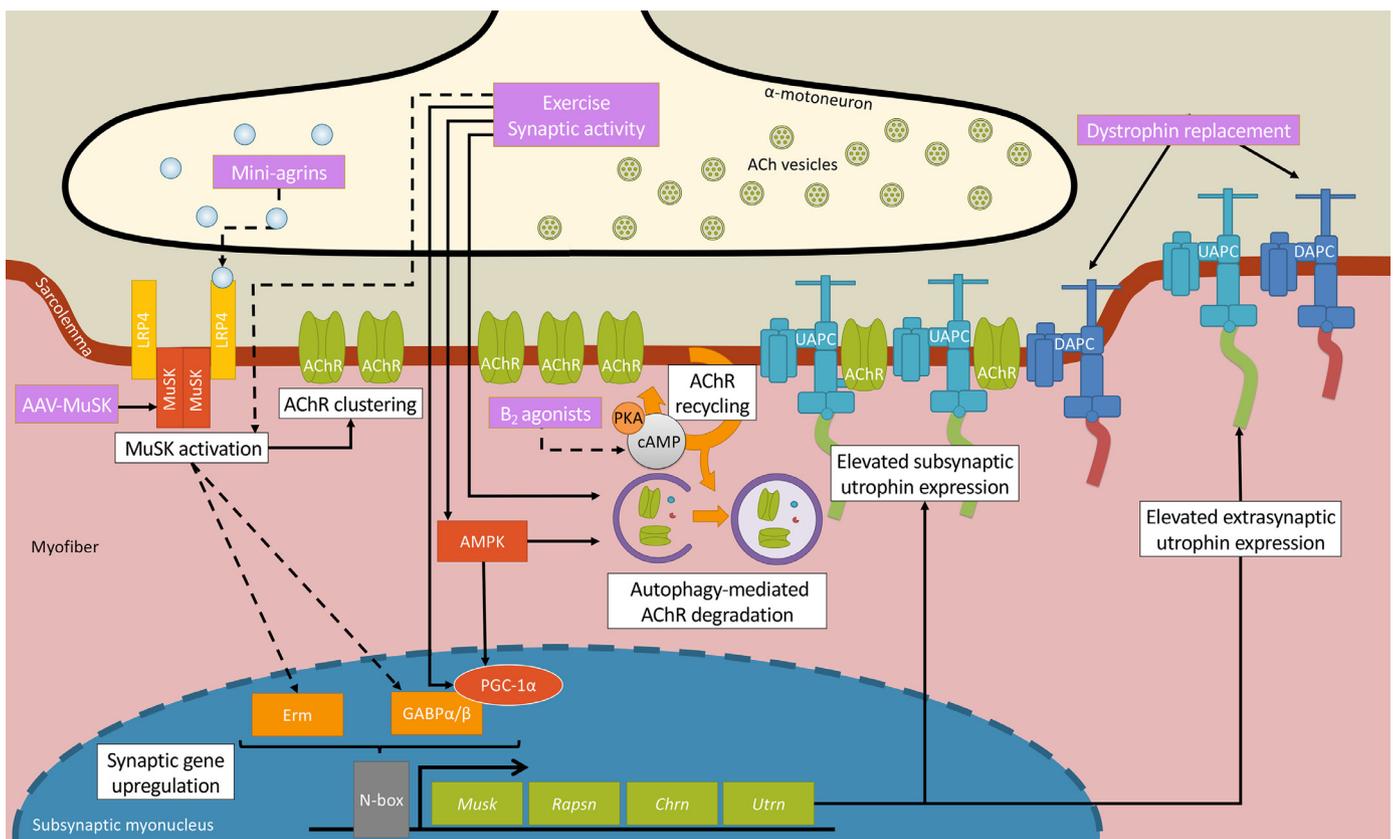
Fig. 2. Therapeutic strategies targeting the NMJ in dystrophic muscle. Several recent pre-clinical studies have provided mechanistic insights into potential therapeutic approaches targeting the NMJ in DMD. Adeno-associated virus (AAV)-MuSK administration improves the dystrophic phenotype by stimulating low-density lipoprotein receptor-related protein 4 (LRP4)/MuSK activation, thereby enhancing AChR clustering and driving gene expression in subsynaptic (also known as fundamental) myonuclei. In particular, downstream of MuSK are the transcriptional activators Ets variant 5 (Erm) and GA-binding protein (GABPα/β; also known as nuclear respiratory factor 2, or NRF-2) that bind to the N-box response element found in numerous synaptic genes, including *Musk, Rapsn, Utrn,* and *Chrn.* Similarly, mini-agrin treatment likely improves dystrophic NMJ biology through LRP4/MuSK activation, however this has yet to be directly demonstrated. Elevating neural activity, for example via a prescribed chronic exercise program, evokes synaptic gene expression by stimulating AMP-activated protein kinase (AMPK)/peroxisome proliferator-activated receptor ɣ coactivator-1α (PGC-1α)/GABPα/β signalling. The chronic induction of *Utrn* expression results in augmented utrophin and UAPC content throughout the myofiber, most notably extrasynaptically along the sarcolemma where it serves to functionally compensate for the lack of dystrophin. Exercise and AMPK also elicit autophagy-mediated AChR recycling, a complementary molecular pathway that beneficially remodels the dystrophic NMJ. β2 adrenergic agonists increase cAMP/protein kinase A (PKA) signalling and normalize AChR recycling and may correct NMJ morphology in dystrophic animals. Lastly, dystrophin replacement strategies, such as mini-dystrophin, have also been shown to improve the stability and function of the NMJ, at least in part via rescue of dystrophin-associated protein complex (DAPC) expression. Solid lines indicate established connections between events and dashed lines refer to potential linkages between steps.

functional abnormities, and by extension mitigate disease progression and severity in dystrophic muscle. A summary of these NMJ-modifying gene expression and signalling cascades are presented in Fig. 2. A current challenge is to translate these pre-clinical data into effective treatments for DMD patients. Continued evaluation of neuregulin/ErbB, Wnt/β-catenin, and Hippo/Yes-associated protein pathways may reveal novel mechanisms for therapeutic pursuit in muscular dystrophy. Additionally, low-intensity, rationally-prescribed (e.g., limiting stressful eccentric contractions) exercise-induced synaptic activity is a safe, accessible, and low-cost behavioural strategy well known to enhance NMJ biology in health conditions that mimic in some ways the dystrophic pathology, such as advanced ageing. However, further research is required to resolve the molecular mechanisms of exercise in the DMD context, particularly with respect to the NMJ. As more practical dystrophin-based gene, cell, and small molecule therapies with better efficacy continue to emerge, we anticipate their correction of NMJ biology as part of a broader improvement in the dystrophic pathology.

In conclusion, the continued examination of the NMJ in DMD will expand our basic understanding of neuromuscular biology. This information may be useful for designing NMJ-targeted drug or behavioural strategies to address the dystrophic pathology and other disorders of the neuromuscular system.

6. Search strategy and selection criteria

Data for this review were identified by searches of PubMed and Google scholar using the search terms ("DMD") AND ("NMJ", "neurotransmission", "synaptic genes", OR "therapies"). References from relevant articles were also manually sought after. All articles referenced are academic and peer reviewed. A preference was given to articles published recently.

Author contributions

SYN and VL conceptualized the paper. SYN wrote the manuscript, constructed the tables, and prepared figures. VL supervised the writing and editing of the manuscript. Both authors have read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors have nothing to disclose.

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