Triadopathies: An Emerging Class of Skeletal Muscle Diseases

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Abstract The triad is a skeletal muscle substructure responsible for the regulation of excitation–contraction coupling. It is formed by the close apposition of the T-tubule and the terminal sarcoplasmic reticulum. A rapidly growing list of skeletal myopathies, here referred to as triadopathies, are caused by gene mutations in components of the triad. These disorders, at their root, are caused by defects in excitation contraction coupling and intracellular calcium homeostasis. Secondary abnormalities in triad structure and/or function are also reported in several muscle diseases, most notably certain muscular dystrophies. This review highlights the current understanding of both primary and secondary triadopathies, and identifies important concepts yet to be fully addressed in the field. The emphasis of the review is both on the pathogenesis of triadopathies and their potential treatment.

Key Words Excitation–contraction coupling · RYR1 · triad · congenital myopathies · malignant hyperthermia

Introduction

The primary function of skeletal muscle is to generate force to initiate and control movement. To accomplish this, muscle utilizes several unique substructures dedicated to force production and regulation [1]. These substructures include the neuromuscular junction (NMJ), the triad, and the sarcomere. As knowledge of the genetic basis of human skeletal myopathies has grown, it has become apparent that many muscle diseases fall into categories based on the fact that they primarily involve alterations of one of these structures. Consideration of muscle diseases by substructural categorization is useful in terms of understanding disease pathogenesis and for therapy development. This review focuses on the triad and disorders that affect triad structure and function—the so-called “triadopathies.”

The triad is an essential skeletal muscle substructure. It represents the close apposition of the transverse tubule (T-tubule) membrane with 2 flanking terminal cisternae of the sarcoplasmic reticulum (SR) (Fig. 1A). The primary role of the triad is to coordinate excitation–contraction coupling (EC coupling). EC coupling is the process by which neuronal input to skeletal muscle [through the release of acetylcholine (ACh) at the NMJ] is transduced into muscle contraction (Fig. 1B). At the triad, membrane depolarization (stimulated by an action potential initiated through ACh binding to ACh receptors at the NMJ) activates dihydropyridine receptors (DHPRs; located in the T-tubule), which, in turn, triggers the opening of the skeletal muscle ryanodine receptor (RyR1) calcium release channels in the terminal SR [2]. Activation of RyR1 results in the release of calcium ions stored in the SR, which then bind to troponin C at the thin filament to initiate actin–myosin

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interactions during the cross bridge cycle. As a result of cross bridge cycling, actin filaments are pulled toward the center of the sarcomere, the sarcomere shortens, and the muscle contracts and generates force. The process is finally reversed upon cessation of calcium release following membrane repolarization and the subsequent reuptake of calcium into the SR through the activity of SR calcium ATPases (SERCAs) [3]. In addition to EC coupling, the triad also participates in the regulation of calcium homeostasis, which, in turn, regulates key cellular process such as oxidative stress, autophagy, and apoptosis.

Recently, defects in triad structure and function have emerged as important underlying causes and/or contributors to a wide range of human muscle diseases [4]. Broadly speaking, alterations in the triad can be grouped into primary “triadopathies”—genetic myopathies where the primary pathogenic mechanism involves an alteration in triad function—and diseases with secondary triad defects. Here we review key features of both the primary triadopathies (Table 1) and disorders in which triad dysfunction plays a key secondary role in the disease process (Table 2). The discussion highlights the critical role of the triad in muscle disease, as well as the development of several potential therapeutic targets and interventions based on triad pathology.

Primary Triadopathies

Malignant Hyperthermia Susceptibility

What Is It?

Malignant hyperthermia (MH) is a pharmacogenetic disorder in which susceptible individuals develop a catastrophic hypermetabolic response to a subset of anesthetics [5, 6]. The classic triggering agents are the inhaled volatile gases (halothane, isoflurane, sevoflurane, etc.), either alone or in combination with a depolarizing muscle relaxing agent like succinylcholine [7, 8]. The presenting signs and symptoms occur either during or just after general anesthesia, and include muscle rigidity (particularly masseter muscle rigidity), rapidly increasing body temperature, respiratory and metabolic acidosis, and cardiac rhythm changes. Muscle breakdown is also often detected through observation of elevated creatine kinase levels and/or myoglobinuria. The consequences of MH are dire, and death is the uniform outcome if untreated. Even with treatment, significant complications commonly occur, affecting 25% of MH survivors [9]. These include renal and/or cardiac failure, coma, pulmonary edema, and disseminated intravascular coagulation. The estimated incidence of MH is between 1:3000 and 1:16,000 anesthetics [10].

MH susceptibility (MHS) is the condition wherein a person has an underlying risk of developing MH. Those with previous MH reactions are considered to have MHS for future

Fig. 1 Structure and function of the triad. (A) The triad is a structure formed by the interface between the T-tubule and 2 portions of the sarcoplasmic reticulum (SR). It is normally seen on electron microscopy of longitudinal sections as a triplet of structures (arrows) between myofibrils and slightly offset from the Z-line. In triadopathies such as myotubular myopathy (MTM), the triad may be absent or disorganized. (B) The triad is a critical structure in the process of excitation–contractions coupling whereby electrical impulses (arrows) travel down in the membrane and into the T-tubules. Interaction between the dihydropyridine receptor (DHPR) in the T-tubule and the ryanodine receptor (RYR) in the SR produces the release of calcium from the SR into the sarcoplasm. This calcium then participates in a variety of cellular processes, including, especially, muscle contraction

anesthetics. However, this term is most usefully applied to individuals who have not had an MH reaction but who either 1) carry a mutation in either *RYR1* or *CACNA1S* that is associated with the development of MH (see below); and/or 2) have a positive diagnostic contracture test (caffeine–halothane contracture test in North America or *in vitro* contracture test in Europe). These contracture tests have high sensitivity (false negative rate approaching 0%) with very good specificity (false positive rate between 10% and 20%). Interestingly, not all individuals who have MHS will develop MH, even after exposure to an established triggering anesthetic.

Table 1 Primary triadopathies

Disorder	Gene	Relationship to triad
Malignant hyperthermia susceptibility	<i>RYR1</i> <i>CACNA1S</i>	Triad hypersensitivity to triggering agents; excessive calcium release from RyR1
<i>RYR1</i> -related myopathies	<i>RYR1</i>	Aberrant EC coupling due to reduced RyR1 function
Centronuclear myopathies	<i>MTM1</i> <i>DNM2</i> <i>BIN1</i> <i>RYR1</i> <i>TTN</i>	Abnormal triad structure and impaired EC coupling. Exact mechanism(s) unclear. May involve aberrant tubulogenesis and/or abnormal membrane recycling
Native American myopathy	<i>STAC3</i>	Abnormal EC coupling. Malignant hyperthermia susceptibility. <i>STAC3</i> may serve as a linker between DHPR and RyR1
Tubular aggregate myopathy	<i>ORAI1</i> <i>STIM1</i>	Abnormal SOCE. Tubular aggregates likely SR-derived

EC=excitation–contraction; RyR1=ryanodine receptor; DHPR=dihydropyridine receptor; SOCE=store-operated calcium entry; SR=sarcoplasmic reticulum

What Causes It?

MH is a pharmacogenetic disorder. The syndrome involves a proper inciting stimulus (i.e., exposure to inhaled anesthetic) occurring in an individual with a susceptible genetic background. Mutations in two genes encoding triad proteins are well established as causes of MHS. The most common genetic change, occurring in >70% of individuals with genetically solved MH, is mutation in the skeletal muscle ryanodine receptor (*RYR1*) [6, 11, 12]. Mutations in *RYR1* associated with MH are almost always heterozygous missense changes that alter protein function [13]. The mutations are inherited in an autosomal dominant fashion. The other characterized cause of MH (occurring in 1% of genetically solved cases) are autosomal dominant mutations of the L-type calcium channel of the T-tubule (*CACNA1S*) [14]. Of note, MHS is genetically heterogeneous, and additional genetic causes are suspected, both from linkage studies that exclude *RYR1* and *CACNA1S* and from direct genetic testing where mutations in the *RYR1*

and *CACNA1S* genes are not present in individuals with confirmed MHS [12]. It is currently estimated that approximately 30% of individuals with MHS do not have a known genetic basis. Ongoing studies using next generation sequencing approaches are likely to identify new MH causative genes in the near future.

How Is The Triad Involved?

RYR1 encodes the calcium release channel located in the terminal cisternae of the SR. As mentioned in the “Introduction”, RyR1 is a key component of the EC coupling apparatus, responsible for the regulated release of calcium from the SR. MH-associated mutations in *RYR1* destabilize the closed state of the channel and result in calcium release channels that are hypersensitive to activation by triggering agents. In other words, MH mutant channels release an excessive amount of calcium in the setting of inciting stimuli (particularly inhaled volatile anesthetics). In turn, the uncontrolled calcium release

Table 2 Secondary triadopathies

Disorder	Gene(s)	Relationship to Triad
Myotonic dystrophy type I	<i>DMPK</i> (CTG repeat expansion in 3'UTR)	Abnormal splicing of key triad gene products (RyR1, DHPR, and BIN1), leading to abnormal EC coupling and altered muscle histopathology
Dystrophinopathies	<i>DMD</i>	Hypemitosylated RyR1 leading to chronic channel “leakiness”
Sarcoglycanopathies	<i>SGCA</i> , <i>SGCB</i> , <i>SGCD</i> , and <i>SGCG</i>	Hypemitosylated RyR1 leading to chronic channel “leakiness”
Dysferlinopathies	<i>DYSF</i>	Dysferlin is a T-tubule component. May stabilize T-tubule and/or chaperone triad proteins
Calpainopathies	<i>CAPN3</i>	Partial localization to the triad. Loss of CAPN3 associated with reduced RyR1 expression
<i>SEPN1</i> -related myopathies	<i>SEPN1</i>	Histopathology similar to <i>RYR1</i> -related myopathies. Direct interaction with RyR1
<i>CCDC78</i> -associated myopathy	<i>CCDC78</i>	Partial localization to the triad. Mutation associated with RyR1 accumulation and abnormal triad structure

UTR=untranslated region; RyR1=ryanodine receptor; DHPR=dihydropyridine receptor; BIN1=bridging integrator 1; EC=excitation–contraction; CAPN3=calpain-3

triggers massive muscle contraction and hypermetabolism, which leads to severe muscle breakdown and the pathologic features associated with an MH crisis (hyperthermia, cyanosis, hyperkalemia, cardiac rhythm disturbances, kidney damage, etc.). Most mutations in *CACNA1S* associated with MHS are thought to also enhance RyR1 sensitivity to activation [14, 15]. *CACNA1S* is the pore-forming subunit of the DHPR, which, under physiologic conditions, functions both as an L-type calcium channel and as a voltage sensor for activation of RyR1 during EC coupling. MH mutations in *CACNA1S* increase RyR1 sensitivity to activation by triggering agents, which then results in excessive calcium release from RyR1.

How Is The Disorder Treated?

The standard therapy for an MH reaction is discontinuation of the triggering agent, whole body cooling, and rapid administration of dantrolene [6, 9]. If given promptly, dantrolene is very effective in minimizing the consequences of MH. The exact mechanism of action for dantrolene is uncertain. Current theories support the concept that dantrolene reduces RyR1 calcium flux and myoplasmic calcium overload, thus limiting the uncontrolled muscle contractions associated with an MH reaction. However, the precise mechanism(s) by which dantrolene accomplishes this is the subject of continued research.

What Are Key Current Issues Related To The Disorder?

Much current effort related to MHS surrounds attempts to identify additional genetic causes for the disorder. Initial efforts using whole exome sequencing have largely resulted in validation of *RYR1* mutations as the primary cause of disease in the majority of cases [11, 16]. These studies have also revealed many *RYR1* variants of unknown significance that require further validation in order to be listed as pathogenic. How to establish pathogenicity for such variants is challenging and is an area of active research. Another topic of interest is the overlap between MHS and other conditions associated with either hyperthermia (e.g., heat stroke) or excessive muscle damage (rhabdomyolysis) [17]. There are several individual case reports that suggest that at least a fraction of these conditions are associated with mutations in *RYR1*. A comprehensive analysis of 39 families with unexplained myalgias and exertional rhabdomyolysis identified causative *RYR1* mutations in 14 of the families [18, 19]. These results are suggestive of an important overlap between these conditions and MH, though additional systematic analyses are needed, and the potential clinical value of dantrolene within these settings has also not been adequately studied.

RYR1-related Myopathies

What Are They?

RYR1-related myopathies are a diverse group of conditions with varied clinical and histopathologic presentations that are caused by mutations in *RYR1* [20]. While MHS (discussed above) is also caused by mutations in *RYR1*, the term *RYR1*-related myopathies generally refers to disorders associated with persistent/chronic muscle dysfunction. These include several subtypes of congenital myopathy: central core disease [21], multiminicore myopathy [22], centronuclear myopathy (CNM) [23], congenital fiber-type disproportion [24], and core-rod myopathy (Fig. 2). Patients with these disorders experience a broad range of clinical symptoms and disease severity. Common features include eye muscle weakness (ophthalmoparesis), extremity muscle weakness, and joint contractures [25]. Patients also frequently exhibit various dynamic muscle symptoms, including MHS, periodic

Fig. 2 Pathological findings associated with triadopathies. (A–D) Representative histopathologic changes in specific ryanodine receptor (*RYR1*)-related myopathies. Micrographs depict (A) centronuclear myopathy (CNM), (B) multiminicore disease (MMC), and (C, D) central core disease (CCD). (E–I) Histopathologic images from cases of myotubular myopathy caused by myotubularin (*MTM1*) mutations. Depicted are (E) myofiber hypotrophy, (F) mitochondrial mislocalization, and (G) necklace fibers. (H) Characteristic radial strands, as typically seen in CNM due to *DNM2* mutation. (I) Electron micrograph of tubular aggregates from an individual with tubular aggregate myopathy. Stains shown on light microscopy images are either hematoxylin and eosin (red/blue) or nicotinamide adenine dinucleotide (blue). Bar=40 μ m for light microscopy and 2 μ m for electron microscopy images

paralysis [26], exertional rhabdomyolysis [19], exercise intolerance, exercise-related myalgias, and fatigue [27]. As with MHS, these dynamic conditions can also exist in the absence of overt muscle weakness. Lastly, there are also particular clinical syndromes that are associated, at least in part, with *RYR1* mutations; these include King–Denborough syndrome (KDS) and late-onset axial myopathy [28, 29].

What Causes Them?

The unifying feature of these conditions is that they are all caused by mutations in *RYR1*. Heterozygous mutations (either autosomal dominantly inherited or *de novo*) are most commonly associated with central core disease and are seen with a very broad range of clinical symptomatology (*de novo* mutations typically being more severe than inherited) [21, 25]. Recessive mutations are most frequently encountered in other myopathic subtypes (particularly minicore myopathy and CNM), and are more prevalent in cases with a severe clinical picture [25, 30]. Recessive mutations are also more commonly associated with external ophthalmoplegia, a finding rarely observed with dominant/*de novo* mutations [25, 30].

How Is The Triad Involved?

In general, most *RYR1*-related myopathies are thought to result from *RYR1* mutations that alter SR calcium release channel function [31]. This can take the form of either reduced RyR1 expression (e.g., with a nonsense or frameshift mutation) or altered channel function (as seen with missense mutations). The ultimate consequence of these changes is impaired EC coupling and diminished muscle force generation, which ultimately leads to muscle weakness. Some mutations are in known functional domains of RyR1 and result in predictable alterations in regulated calcium release from the SR. However, many mutations are not so obviously correlated with clear reduction in calcium release, and their pathogenicity is far less clear. Of note, in many recessive cases, RyR1 expression is markedly reduced and this is thought to be a key mechanism of disease that correlates with disease severity [30].

How Are These Disorders Treated?

At present, there are no rigorously studied therapies for *RYR1*-related myopathies. Case reports exist for a few candidate therapies and additional pharmacologic strategies have been proposed based on preclinical studies. Salbutamol, a β -adrenergic agonist, was shown to improve both muscle strength and respiratory function in a pilot trial of 13 patients with core myopathy [32]. The specific mechanism of action for the drug in this setting is not known. Thus, additional clinical studies are needed to determine true potential efficacy

of this therapy. There is a single case report of improvement in motor function with oral dantrolene in a patient with central core disease and mild myopathy [20]; however, there are also descriptions of dantrolene causing increased muscle weakness after administration. Therefore, the clinical utility of dantrolene outside of MH is unclear. Response to dantrolene may ultimately depend on the specific *RYR1* mutation and that mutation's effect on the dynamics of calcium release; this is clearly an area of much needed additional research, likely at the preclinical level.

In terms of new drug development, perhaps the most promising “lead” at present is *N*-acetylcysteine (NAC). In a collaborative study [33], we (Dowling) uncovered increased cellular stress and aberrant oxidative stress in a zebrafish model of *RYR1*-related myopathy and in myotubes from patients with *RYR1* mutations. Exposure of the zebrafish mutants to NAC improved muscle structure and motor endurance. Importantly, treatment of patient myotubes with NAC decreased pro-oxidant-mediated cell death. Consideration of a clinical trial to test the efficacy of NAC in *RYR1*-related myopathy patients is underway. However, as oxidative stress is likely a “secondary” consequence of impaired calcium handling due to *RYR1* mutation, therapy with NAC does not address the primary problem of altered EC coupling in these disorders. Therefore, other strategies are clearly needed. One such pharmacologic strategy would be chemical modulators that improve the ability of RyR1 to release calcium. An example of a potential therapeutic is the Rycal class of drugs, which stabilize RyRs to “improve” their ability to release calcium [34]. Rycals are under consideration for the treatment for heart failure, but have yet to be tested in preclinical models of *RYR1*-related myopathies.

Another set of therapies under consideration are gene editing strategies. *RYR1* is too large for conventional gene therapy approaches, so other techniques must be utilized. In principal, *de novo*/dominant *RYR1* mutations are candidates for targeting by exon skipping, allele-specific gene silencing or trans-splicing. Such approaches, if used to produce a loss of function allele in the setting of a heterozygous mutation, could be of potential benefit because haploinsufficiency for *RYR1* does not cause overt clinical symptoms. Of course, the challenge would be targeting only the mutated allele. One example of a gene-based approach was provided by our group (Dirksen) using allele-specific silencing in 2 mouse models of central core disease [35]. We developed small interfering RNA specific to the heterozygous mutation in the mouse (Y524S in one model and I4898T in another) to preferentially knock down the mutant allele and restore EC coupling in adult mouse muscle.

What Are Key Current Issues Related To The Disease?

With the application of next generation sequencing technology to clinical neuromuscular diagnostics, the identification of

patients with *RYR1* mutations has expanded exponentially. It is now clear that *RYR1*-related myopathies are the most common nondystrophic muscle conditions of childhood and that the clinical spectrum for the disorders is vast [36, 37]. One important issue to emerge is how to interpret identified sequence variants in *RYR1* that are of uncertain pathogenicity [11, 16]. AS *RYR1* mutations are associated with such a broad range of disease, most novel variants identified in *RYR1* have the potential of causing disease. Thus, the challenge for the clinical and research communities is to develop tools and algorithms that will aid and assist in the clinical interpretation of newly identified *RYR1* variants.

Another interesting issue is the clinical and histopathologic variability associated with *RYR1* mutations, both among unrelated individuals and within families with the same mutation [25]. It is not clear why different histopathologic phenotypes (e.g., core myopathy and CNM) emerge and whether there is any special significance (either at the pathogenic level or in terms of clinical presentation and outcome) with regard to the histological subtype of myopathy a given patient manifests. Also, the factors that account for the variable expressivity of clinical disease among individuals with the same mutation—a variability that can be relatively dramatic in some instances—have not been explored in depth, but may represent both external factors and gene-based modifiers. One explanation for interfamilial clinical variability, at least in some cases, is the presence of additional (second-site) *RYR1* mutations segregating within the family, as has been described by Klein et al. [25].

A final important consideration is the relative lack of therapeutic candidates for *RYR1*-related myopathies. Other than NAC, no treatments are currently being considered for clinical testing. One barrier to treatment development is the relatively small number of preclinical models, and the difficulty presented by the phenotypes of those models. The *Ryr1* knockout mouse (“dyspedic”) dies at birth, and thus is not suitable for most *in vivo* studies [38, 39]. The available RyR1 “knockin” mouse models, in which identified disease mutations are engineered into the mouse genome, model these conditions well and have provided a wealth of information about MHS, core myopathy, and RyR1 pathophysiology [40]. However, because of cost and low inherent throughput in working with mice, these models have not been ideal for drug discovery. In addition, no mouse model exists that recapitulates a non-MH/CCD-related *RYR1* myopathy. Recently, the zebrafish model has been utilized as an excellent preclinical vertebrate model for therapy identification [39]. This is well illustrated by the fact that NAC was identified as a potential therapeutic intervention using this system [33]. Additional studies using the existing zebrafish model (a recessive loss of expression mutant in *ryr1b* that most closely models *RYR1*-related CNM) will hopefully yield additional candidates. There is also a need to develop additional zebrafish mutants

designed to reflect the wider range of clinical and pathologic presentations of *RYR1*-related myopathies.

CNMs

What Are They?

CNMs are a subset of the broader category of muscle diseases called congenital myopathies [4]. CNMs are unified by common histopathologic features on muscle biopsy, most notably the presence of large, centrally located nuclei in at least 10% (and often in excess of 25%) of muscle fibers [41]. CNMs can be further subdivided into groups based on genetic cause [42]. The most common genetic cause is mutations in myotubularin (encoded by *MTM1*). Patients with *MTM1* mutations are said to have myotubular myopathy [43]. *MTM1* is an X-linked gene and mutations primarily affect males. In its classic form, myotubular myopathy is an extremely severe disorder characterized by weakness and respiratory failure that presents at birth [44]. A significant percentage of individuals with myotubular myopathy do not survive the first year of life, and those that do exhibit lifelong disabilities, including dependence on ventilators, wheelchairs, and gastrostomy tubes.

RYR1 mutations are likely the second most common cause of CNM [22, 30]. The clinical presentation is varied, though individuals usually present with significant symptoms in childhood. Some individuals with *RYR1*-related CNM present with severe symptoms that phenocopy those seen with *MTM1* mutations. The next most common cause of CNM is mutations in *DNM2*, which present as either *de novo* or dominant mutations [45, 46]. There is a mild adult onset form of *DNM2*-related CNM, usually caused by mutations within the middle domain of *DNM2*. There is also a severe childhood form that is often associated with mutations in the *DNM2* PH domain. Two rare causes of CNM are mutations in *BIN1* (bridging integrator 1, also called amphiphysin 2) and Titin (*TTN*), both of which exhibit an autosomal recessive form of inheritance [47, 48]. Patients with *TTN* mutations also may present with cardiomyopathy. In addition, approximately 25% of cases of CNM are genetically unsolved [49].

What Causes It?

As mentioned above, there are 5 known genetic causes of CNM. Mutations in *MTM1*, *RYR1*, and *BIN1* are all recessively inherited and thought to result from a loss of gene function. *TTN* mutations are also recessive, though the consequences of mutations in the *TTN* gene product remain unclear. At least a part of the *TTN* protein is not translated in some patients with *TTN*-related CNM [48]. The pathomechanism of *DNM2* mutations has been somewhat perplexing [50]. All *DNM2* mutations are either dominant or *de novo* missense mutations, suggesting a potentially toxic gain-of-function effect

produced by the mutations. This hypothesis is supported by the observation that nonsense mutations in *DNM2* that result in haploinsufficiency do not cause disease [51]. However, several studies have shown that loss of basic *DNM2* functions (such as the regulation of endocytosis) occurs with some mutations, suggesting that loss of function may also contribute to the disease mechanism.

How Is The Triad Involved?

The identification of triad abnormalities as a key driver of CNM pathogenesis is a relatively recent discovery. Our group (Dowling) identified structural changes in the triad and altered EC coupling in a zebrafish model of myotubular myopathy [52]. In the same study, we observed similar structural alterations in the triad in biopsies from patients with *MTM1* mutations. These findings were soon confirmed by the description of similar changes in the mouse model of myotubular myopathy [53]. Investigation of muscle biopsies from CNM patients with *BIN1* and *DNM2* mutations [54–57], as well as supporting data from animal models of these diseases, revealed that triad changes also represent an important aspect of these CNMs. Lastly, the identification of mutations in *RYR1* as a cause of CNM cemented the link between triad dysfunction and this congenital muscle disease.

The role of *RyR1* dysfunction in muscle disease is discussed in the previous section; the reason why certain *RYR1* mutations cause the distinctive histopathologic changes associated with CNM is not clear. *BIN1* was identified as a modulator of T-tubule biogenesis well before mutations in this gene were linked to CNM [58]. *BIN1* is a BAR (Bin1-Amphiphysin-RVS167) domain-containing protein that regulates and modifies membrane curvature; loss of *BIN1* function is hypothesized to alter T-tubule formation and maintenance. *DNM2* has multiple cellular functions, many of which relate to its ability to regulate membrane trafficking. Moreover, *DNM2* interacts directly with *BIN1*. Thus, a leading hypothesis for how *DNM2* mutations affect the triad is that mutant *DNM2* proteins alter/prevent normal *BIN1* function(s). The reason why mutations in *MTM1* cause defects in triad structure is not clear. *MTM1* is a lipid phosphatase that regulates the levels of the phosphoinositides *PI3P* and *PI3,5P2*. While *MTM1* is localized to the triad, evidence linking alterations in levels of *PI3P* and/or *PI3,5P2* at the triad is less robust. Thus, the mechanism underlying the association between loss of *MTM1* function and defective triad structure is still uncertain. Lastly, other than the histopathology, there is no known link to date between *TTN* and the triad. Given the enormous size of *TTN* and its numerous protein–protein interactions, one possibility is that CNM-related mutations in *TTN* interfere with or alter other proteins that more directly regulate the triad. One intriguing possibility is calpain-3, the expression of which is diminished in the muscle biopsies of patients with *TTN*-

related CNM [48]. A potential association between calpain-3 and the triad is discussed in the “Secondary Triadopathies” section.

It is important to note that pathways and structures other than the triad are likely also affected in CNMs. Other than *RYR1*, all of the known CNM-associated proteins have well-documented functions in processes unrelated to the triad. For example, *MTM1* has been implicated as a regulator of autophagy and of the desmin intermediate filament network [59–61]. In addition, loss of *MTM1* in both zebrafish and mouse models is associated with alterations of the NMJ (see below) [62, 63].

How Is The Disorder Treated?

There have been several exciting therapeutic advances discovered using preclinical models of CNM. By far the most promising is related to myotubular myopathy. An international collaborative group demonstrated that gene therapy with *MTM1* not only prevents, but also reverses, the phenotype of *Mtm1* knockout mice [64]. This same group also demonstrated the efficacy of local adenoassociated virus–*Mtm1* infusion as a treatment for dogs with *Mtm1* mutations. Another parallel advance has been the use of enzyme replacement therapy with *MTM1* protein, where a pilot study using locally administered *MTM1* improved the disease phenotype in *Mtm1* knockout mice [65]. Both *MTM1* gene and protein therapies are poised for testing in patients via clinical trials.

Enhancing signaling at the NMJ is another therapeutic strategy being developed for both myotubular myopathy and *DNM2*-related CNM. In zebrafish models of both conditions, treatment with edrophonium (an acetylcholinesterase inhibitor) significantly improved motor function [63, 66]. Similarly, in the mouse model of myotubular myopathy, treatment with pyridostigmine (another acetylcholinesterase inhibitor) significantly improved exercise tolerance [62]. At present, there are also several case reports of individuals with *MTM1*, *DNM2*, and, very recently, *RYR1* mutations exhibiting positive responses to pyridostigmine, though this has not been systematically studied [27, 63, 66]. Given that there is evidence for structural abnormalities of the NMJ in models of at least some of the CNMs [62, 66], it is likely that pyridostigmine works by addressing this aspect of disease pathology, and not through direct effects on the triad.

What Are Current Issues Related To The Disease?

One of the major unanswered questions regarding CNM is the primary cause(s) for the signature pathogenic features (i.e., central nuclei) of the disorder. Because the gene products of several CNM genes, particularly *MTM1* and *DNM2*, regulate multiple cellular processes, it was initially assumed that the central nuclei resulted from a disrupted process independent

of triad pathology. However, the fact that *RYR1* mutations, the gene product of which exclusively functions within the triad, also cause the disorder suggests that triad disturbance may be sufficient to trigger central nucleation. Interestingly, work in the *Mtm1* knockout mice has shown that triad changes precede the presence of central nuclei [53], lending additional evidence to the hypothesis that disruption of normal triad structure/function leads to mislocalization of nuclei. Additional studies are needed to elucidate the specific molecular changes and sequence of events that underlie this phenomenon.

Native American Myopathy

What Is It?

Native American myopathy (NAM) is a rare inherited muscle disease that, at present, has only been reported in the Lumbee Native Americans of North Carolina [67]. The cardinal clinical features of the condition are mild muscle weakness, ptosis, myopathic facies, short stature, kyphoscoliosis, and a distinctive gait. Susceptibility to MH is also associated with this myopathy.

What Causes It?

NAM is caused by a recessive missense mutation (W284S) in *STAC3* (68). *STAC3* (SH3 and cysteine rich domain 3) encodes an approximately 50-kDa protein with SH3 and cysteine-rich domains. The precise mechanisms by which mutations in *STAC3* result in myopathy are currently unclear.

How Is The Triad Involved?

STAC3 localizes to the triad where it interacts with both the DHPR and RyR1. Loss of *Stac3* in either zebrafish or mouse disrupts EC coupling [68, 69]. A current hypothesis is that *STAC3* serves as either an essential linker or as a chaperone for the mechanical communication between the DHPR and RyR1.

How Is It Treated?

There are currently no treatments for NAM, nor any putative therapeutic strategies. Individuals with NAM who experience an MH reaction are treated with dantrolene.

What Are Some Current Issues Related To The Disease?

It will be of interest to see whether *STAC3* mutations are confined to the Lumbee population or are more widely observed. It is possible that the clinical phenotype of individuals outside the Lumbee population will be different. One potential

allelic phenotype is KDS, a condition that shares many similarities to NAM including weakness, an unusual gait, and MH susceptibility. To date, only *RYR1* mutations have been found as a cause KDS, though they do not account for all cases [28].

Another question related to *STAC3* is why individuals with NAM are susceptible to MH. Loss of *STAC3* causes an impairment in EC coupling and a reduction in regulated SR calcium release [68, 69]. *STAC3* complementary DNA containing the NAM mutation does not fully rescue the *stac3* knockout phenotype in zebrafish [68], suggesting that, at least in part, it may represent a hypomorphic allele. It is therefore perplexing how an apparent reduction in EC coupling caused by *STAC3* mutation is associated with MH, a phenotype usually associated with hyper-responsive calcium release.

Tubular Aggregate Myopathy

What Is It?

Tubular aggregate myopathy is a mild, slowly progressive muscle condition that often begins in early childhood [70]. It features prominent myalgias and muscle cramping. It is also a component of a broader syndrome called Stormorken syndrome, which, in addition to tubular aggregates, also features hematopoietic abnormalities [71]. The condition is diagnosed by muscle biopsy where aggregates of hexagonal tubules are seen by electron microscopy. Of note, tubular aggregates can also be seen as a secondary pathologic change in other muscle diseases.

What Causes It?

Both tubular aggregate myopathy and Stormorken syndrome are caused by mutations in either *STIM1* or *ORAI1* [71–73]. The *STIM1* and *ORAI1* mutations described thus far are dominant and result in constitutive activation of the respective gene products. Of note, recessive loss of function expression mutations in both genes are associated with immunodeficiency syndromes [74].

What Is The Relationship To The Triad?

ORAI1 is the pore-forming component of the store-operated calcium release-activated calcium channel, while *STIM1* is the calcium-sensing component of the channel [75]. *ORAI1* and *STIM1* interact with each other upon store depletion, and both proteins are involved with the process of store-operated calcium entry (SOCE) [76, 77]. SOCE is a critical aspect of the regulation of calcium within the muscle fiber and it is intimately linked with EC coupling. In fact, enhanced SOCE activation has been shown to be an important part of the pathogenesis of MH [78, 79]. *STIM1* and *ORAI1* are found at the triad, but are distinct from the DHPR/RyR1 interface

[75]. The tubular aggregates that are the pathognomonic histopathologic abnormality of the disorder are likely composed of SR-derived membranes [80].

How Is It Treated?

There are currently no treatments for the myopathic symptoms associated with myopathies due to either *ORAI1* or *STIM1* mutations.

What Are Some Current Issues Related To The Disease?

Tubular aggregates are the defining microscopic observation in patients with tubular aggregate myopathy. However, tubular aggregates are seen in a broader range of myopathic states. For example, experimental evidence has linked accumulation of tubular aggregates with muscle aging [81]. Therefore, it is possible that tubular aggregate myopathy may represent a premature aging or senescence syndrome. Future studies are needed to address this hypothesis. Another critical question requiring additional experimentation is the mechanism by which abnormalities in SOCE lead to the formation of tubular aggregates.

Secondary Triadopathies

Abnormalities at the triad, usually in its function and not its structure, have also been described as secondary pathomechanisms in several muscle diseases. This section will briefly review these associations and discuss the potential implications for disease treatment.

Myotonic Dystrophy Type I

Myotonic dystrophy type I (DM1) is a multisystem disorder that is caused by a CTG repeat expansion in the 3' untranslated region of *DMPK* [82]. DM1 primarily results from impaired alternative splicing of selected mRNA transcripts in tissues in which dystrophin myotonia-protein kinase is expressed. The repeat expansion disrupts splicing by sequestering splicing regulatory factors such as muscleblind and other CUG binding proteins (e.g., CUGBP1) [83]. Splicing of several genes associated with triad function are disrupted in DM1. In particular, splice changes have been described for RyR1, DHPR subunits, and BIN1. The splice changes in RyR1 affect a domain involved with channel inhibition and result in altered/impaired EC coupling [84, 85]. Altered splicing of the DHPR leads to changes in L-type Ca^{2+} channel conductance [86]. Missplicing of BIN1 results in enrichment of an inactive form of the gene product, which, in turn, disrupts T-tubule formation and maintenance, as determined by targeted alteration of the spliced exon in the mouse [87]. The BIN1 splicing

alteration is proposed to be responsible for the central nucleation pattern commonly observed in muscle from patients with DM1 and to contribute to the muscle weakness observed in this disease.

Muscular Dystrophies

Dystrophinopathies

Mutations in dystrophin (*DMD*) cause the allelic conditions Duchenne muscular dystrophy and Becker muscular dystrophy [88]. Dystrophin encodes a massive protein that links the sarcolemmal membrane to the actin cytoskeleton. It also interacts with numerous regulatory proteins. A primary function of dystrophin is to stabilize the muscle membrane during muscle contraction. Loss of dystrophin results in increased membrane fragility, which leads to subsequent damage to the myofiber due to stress-induced membrane microtears. There is also considerable data to suggest that loss of dystrophin alters various signaling pathways (e.g., nitric oxide synthase) [89].

Interestingly, there is evidence that, in addition to its typical plasma membrane localization, dystrophin is also found at the T-tubule [90]. While its role at the T-tubule and in the triad is not well understood, there is evidence of abnormal EC coupling in the most common preclinical model of dystrophinopathy, the *mdx* mouse (which harbors a nonsense mutation in *Dmd*) [91]. There are also data showing that this EC coupling abnormality, at least in part, is due to RyR1 dysfunction. Specifically, Bellinger et al. [92] have shown that RyR1 is hypernitrosylated in the *mdx* mouse, and that RyR1 hypernitrosylation leads to a “leaky” RyR1 channel. Chronic RyR1 “leakiness” is associated with reduced calcium release during EC coupling and contributes to intracellular calcium-mediated toxicity (including enhanced oxidative stress).

Two therapies related to altered RyR1 function and abnormal calcium homeostasis have been tested in the *mdx* mouse model. One is Rycal (mentioned earlier), which is an RyR1 sensitizer that improves stimulus-dependent RyR1 calcium release. Rycals are currently under consideration for the treatment of cardiomyopathy associated with dystrophinopathy, which is thought to be due, in part, to defective RyR2 signaling in the heart [93]. The second therapeutic approach involves the overexpression of SERCA1. SERCA1, a protein primarily localized in the longitudinal SR, is responsible for the reuptake of calcium into the SR and thus the rate of muscle relaxation. Goonasekera et al. [94] recently demonstrated that transgenic upregulation of SERCA1 in *mdx* mice reduced development of the dystrophic muscle pathology in this model.

Sarcoglycanopathies

Sarcoglycanopathies are muscular dystrophies caused by gene mutations in any one of 4 sarcoglycan genes [95]. The

sarcoglycans form a complex at the sarcolemmal membrane that is part of the dystrophin-associated membrane complex. Recessive mutations in sarcoglycan genes cause a phenotype very similar to that seen in the dystrophinopathies. Similar to dystrophin mutations, there is also evidence of impaired EC coupling and altered RyR1 nitrosylation in a mouse model of sarcoglycan deficiency [96]. In addition, in the same study that examined *mdx* mice, Goonasekera et al. [94] showed that transgenic SERCA upregulation improves the dystrophic phenotype in sarcoglycan-deficient animals, again via a mechanism similar to that proposed for dystrophinopathy.

Dysferlinopathies

Dysferlin is a membrane-associated protein primarily implicated in the regulation of membrane repair. Loss of dysferlin function is associated with autosomal recessive limb girdle muscular dystrophy type 2B (LGMD2B) [97]. Dysferlin is also a component of the T-tubule. Its precise function at the T-tubule is not entirely clear, though dysferlin has been shown to stabilize stress-induced calcium signaling in the T-tubule membrane and serves as a chaperone for T-tubule proteins in the triad such as the DHPR [98, 99].

Calpainopathies

Calpain-3 (CAPN3) is a nonlysosomal cysteine protease implicated in the cleavage and/or breakdown of multiple key skeletal muscle proteins. Mutations in *CAPN3* cause autosomal recessive limb girdle muscular dystrophy 2A (LGMD2A), a slowly and variably progressive disorder associated with proximal muscle weakness [100]. Kramerova et al. [101] identified CAPN3 localization at the triad (amongst other places). They showed that *Capn3* knockout in mice was associated with decreased expression of RyR1 in skeletal muscle, as well as with impaired SR calcium release [101]. Based on these results, the authors proposed that impaired triad function was an important aspect of the disease process in patients with LGMD2A. Further work is clearly necessary to strengthen the association and causal links between CAPN3, triad structure/function, and LGMD2A disease pathogenesis associated with CAPN3 mutations. However, the fact that the precise relationship between CAPN3 function and LGMD2A is poorly understood despite much research makes this avenue of exploration worthwhile.

SEPNI-related Myopathies

SEPNI encodes selenoprotein-N, an endoplasmic reticulum-resident protein that is part of the selenoprotein family.

Recessive mutations of *SEPNI* are associated with multimincore myopathy, rigid spine muscular dystrophy, congenital fiber-type disproportion, and Mallory Body myopathy [102]. Loss of SEPNI function is associated with increased oxidative stress in muscle [103], accelerated muscle pathology in the setting of physiologic stressors [104], and with abnormalities in muscle stem cells [105].

SEPNI function, and the diseases that result from *SEPNI* mutations, likely include significant triad involvement. The most compelling evidence for this is the substantial clinical and histopathologic overlap between *SEPNI*- and *RYR1*-related myopathies [106]. In particular, both are associated with prominent axial weakness and are the primary causes of core myopathy. In addition, a direct interaction between RyR1 and SEPNI has been demonstrated [107]. The study that documented this interaction also provided evidence that loss of *Sepn1* in zebrafish is associated with impaired calcium handling. Moreover, as with *RYR1*-related myopathies, there is abundant evidence that *SEPNI* myopathies are associated with enhanced oxidative stress. Furthermore, NAC improves survival in myocytes from patients with *SEPNI* mutations, and thus represents a potential therapeutic intervention for this disease [108]. On the contrary, and despite this evidence, SEPNI expression is largely confined to muscle development and the gene product is not abundantly found in adult mouse muscle [109], making a primary role in triad biology less likely. Additional studies are clearly needed to better delineate a potential link between SEPNI and RyR1 at the triad.

CCDC78-associated Myopathy

Our group (Dowling) recently identified a dominant splice site mutation in *CCDC78* in a family with an unusual congenital myopathy associated with moderate muscle weakness, exercise intolerance, and a combination of cores, protein aggregates, and central nuclei on muscle biopsy [110]. Using a morpholino-based approach, we showed that zebrafish that model the splice site alteration seen in patients exhibit abnormal triad structure and accumulate RyR1 and DHPR aggregates throughout their myofibers. Similar aggregates were also observed in patient muscle biopsies. Given that *CCDC78* localizes, at least in part, to the triad, it is reasonable to speculate that 1) *CCDC78* plays a role in triad structure/function and 2) *CCDC78* mutations act, at least in part, by disrupting EC coupling. Little additional information is currently available regarding the role of *CCDC78* in muscle. A study of *CCDC78* in *Xenopus* identified it as a required component of the deuterosome [111]. How and to what degree this may relate to its function in muscle remains to be understood.

Summary

The triad is a key muscle substructure responsible for controlling muscle contraction. Abnormalities in the triad are an emerging primary cause of an eclectic array of distinct skeletal myopathies called triadopathies. The overall incidence of triadopathies, as well as the breadth of gene mutations associated with the triad, is likely to increase as whole exome and whole genome sequencing approaches are applied to genetically unsolved myopathies. Therapy directly targeted at improving triad structure and function, and particularly at normalizing RyR1 activity, represents a promising treatment strategy for both the primary and secondary triadopathies. New drug discovery is clearly needed, as are new preclinical models to advance mechanistic understanding of disease pathogenesis and to improve therapy identification/development. In this review, we detailed issues related to altered triad stability and function in the triadopathies, highlighting studies that lay the groundwork for future mechanistic and translational research into this interesting and clinically relevant field.

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