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Sodium dysregulation coupled with calcium entry leads to muscular dystrophy in mice

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**Abstract** Duchenne Muscular Dystrophy (DMD) and many of the limb girdle muscular dystrophies form a family of diseases called sarcoglycanopathies. In these diseases, mutation of any of a host of membrane and membrane associated proteins leads to increased stretch induced damage, aberrant signaling, and increased activity of non-specific cation channels, inducing muscle necrosis. Due to ongoing necrosis, DMD follows a progressive clinical course that leads to death in the mid-twenties. This course is slowed only modestly by high dose corticosteroids, which cause a plethora of harsh side effects. Targeted therapies are needed to ameliorate this disease until a more permanent therapy such as replacement of the mutated gene can be routinely performed. Here, we identified sodium calcium exchanger 1 (NCX1) as a potential therapeutic target. We started from the observation that sodium calcium exchanger 1 (NCX1) was upregulated during the necrotic phase of the disease in \( Sgcd^{-/-} \) mice, which have similar pathology and mechanism of disease to boys with \( DMD \). To test the causal effect of NCX1 overexpression on disease, we generated mice that overexpress NCX1 specifically in skeletal muscle. By Western blotting and immunofluorescence, we showed that NCX1 transgenic mice express more NCX1 protein in a similar localization pattern as endogenous NCX1. Sodium calcium exchange activity was also shown to be increased in NCX1 TG mice using an \( \textit{in situ} \) sodium calcium exchange assay. When we examined the histology of NCX1 TG mice, we found that overexpression of NCX1 caused pathologic changes in skeletal muscle of the hindlimb. We then crossed the NCX1 tranogene into \( Sgcd^{-/-}, \ mdx, \) and \( Dysf^{-/-} \) dystrophic backgrounds and found that the disease of these models was increased by the presence of the NCX1 transgene. Interestingly, NCX1 mediated opposing effects in the diaphragm, where NCX1 transgenic overexpression was protective in dystrophic backgrounds. To determine the effect of endogenous NCX1 on dystrophic pathology we deleted \( Slc8a1 \ (NCX1) \) using a muscle specific Cre recombinase and found that deletion of NCX1 was protective to the hindlimb. We next measured resting intracellular sodium and calcium concentrations and found that NCX1 increased resting sodium and
calcium levels. This suggested to us that NCX1 was acting in reverse mode to cause pathology. Thus, we exacerbated reverse mode activity by generating NCX1 TG sodium potassium ATPase heterozygous mice, which have decreased pump function and treated NCX1 TG mice with the NKA inhibitor digoxin. In both cases, we saw a dramatic exacerbation of the NCX1 TG phenotype, strongly suggesting that reverse mode is the mechanism of disease. To translate these findings into a potential therapeutic intervention, we then utilized an FDA approved drug, ranolazine, to treat Sgcd/- mice. Because ranolazine inhibits sodium channels it should enhance forward mode activity of NCX1. Indeed, we found decreased histopathology in mice treated with ranolazine. Together, these data identify a novel mechanism of dystrophic pathology and a promising therapeutic for future study.
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Abbreviations

α, alpha; β, beta; γ, gamma; δ, delta; ; A band, anisotropic band; μ, micro; μg, microgram; μM, micromolar; ATP, adenosine triphosphate; Atp1a2, gene for sodium potassium ATPase alpha 2; BTS, benzyl-p-toluenesulphonamide; Ca2+, calcium ion; cDNA, complimentary deoxyribonucleic acid; C-terminal, carboxy terminal; d, day; DAPI, 4’,6’-Diamidino-2-Phenylindole, Dihydrochloride; DGC, Dystrophin glycoprotein complex; DKO, double knock out; DMD, Duchenne Muscular Dystrophy; dnTRPC6, dominant negative transient receptor canonical C6; dnTRPV2, dominant negative transient receptor potential vanilloid 2; Dysf, dysferlin gene name; EDL, extensor digitorum longus; ER, endoplasmic reticulum; FDB, flexor digitorum brevis; FITC, fluorescein isothiocyanate; g, gram; H&E, hematoxylin and eosin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; H-zone, H is from german word Heller which translates to brighter; I band, isotropic band; kg, kilogram; LGMD, limb girdle muscular dystrophy; L-type calcium channel, the L refers to the long lasting activation of the channel; MAC, mechanically assisted cough; MD, muscular dystrophy; mdx, muscular dystrophy mouse; mg, milligram, Mlc1, myosin light chain 1 gene name; M-line, M is from the German word mittelscheibe, or middle); MPTP, mitochondrial permeability transition pore; Na,K-ATPase, sodium potassium adenosine triphosphatase; Na+, sodium ion; NAPDH, nicotinamide adenine dinucleotide phosphate; Na1.4, sodium channel protein 4 subunit alpha; NCX1 TG, sodium calcium exchanger 1 transgenic; NCX1, sodium calcium exchanger 1; NCX3, sodium calcium exchanger 3; NIV, non-invasive ventilation; NKA, sodium potassium adenosine triphosphatase; N-terminal, amino terminal; NTG, non-transgenic; PBS, phosphate buffered saline; pCA, -log([Ca2+]); PMCA, plasma membrane calcium ATPase; PVDF, polyvinylidene difluoride; RIPA, radioimmunoprecipitation buffer; ROCE, receptor operated calcium entry;
SERCA, sarcoplasmic endoplasmic reticulum calcium adenosine triphosphatase; Sgcd, delta sarcoglycan gene name; SLC8a1, solute carrier 8a1; SOCE, store operated calcium entry; SR, sarcoplasmic reticulum; Stim 1, stromal interaction molecule 1; TEA-CL, tetraethylammonium chloride; TRPC1, transient receptor potential canonical 1; TRPC3, transient receptor potential canonical 3; TRPV2, transient receptor potential vanniloid 2; t-tubule, transverse tubules; WT, wild type; X-ROS, X reactive oxygen species; Z-line, Z is from the German word zwischenscheibe, which roughly translates to “in between” in reference to the I-bands
Chapter 1

Introduction
Skeletal muscle background

The function of skeletal muscle is to contract facilitating movement. Through evolution, muscle has developed specialized characteristics, allowing the same muscle to effect both powerful and precision movements. The primary unit of the muscle is the sarcomere, which is an individual contractile unit. These sarcomeres, repeated thousands of times both in series and in parallel are contained within a huge syncytial cell composed of thousands of myonuclei. Every muscle fiber is surrounded by a basement membrane called the endomesium, which adds mechanical strength to the membrane, and is necessary to withstand contraction. Bundles of muscle fibers are organized into fascicles demarcated by a thicker extracellular matrix called perimysium (Figure 1). All of the fascicles that make up a muscle are surrounded by a thicker layer of extracellular matrix called the epimysium. The modular organization of muscle gives versatility to muscle contraction, allows paths for vessels, and adds stiffness to protect the muscle during movement.

At an ultrastructural level, the muscle fiber is organized into repeating contractile units called sarcomeres. The thin filament, made up of actin and the actin regulatory proteins and the
thick filament, made up mainly of myosin, are the major components of the sarcomere. In healthy muscle, the thin and thick filaments are highly organized into repeating bands that are visible by electron microscopy (figure 2). The ends of the sarcomere, which appear as thin electron dense bands, are termed Z-disks, which are crucial for anchoring the sarcomere to the membrane and subsequently the ECM. Adjacent to the Z-disc is a less electron dense structure made up of the thin filament composed of actin and its regulatory elements termed the I band (254). Located in the center of the sarcomere is a wide electron dense band termed the A band, which represents the region of overlap between the thin and thick filaments. In the center of the A band, is a central electron dense band termed the M-band, which contains M-protein and other cytoskeletal components (1). Flanking the M-band on either side are the H-zones, which represent areas where only the thick filament is present. To provide the ATP fuel required for contraction, large numbers of mitochondria are found between the I bands of adjacent sarcomeres.

Surrounding the contractile apparatus is also a complex membrane structure made of t-tubules and the sarcoplasmic reticulum (SR), which form the calcium release system of the muscle fiber. The SR is a specialized form of endoplasmic reticulum (ER) which is highly enriched in ryanodine receptors and calcium pumps, facilitating storage and rapid coordinated release of calcium. The signal to release calcium is transmitted to the center of the muscle fiber by long invaginations of plasma membrane called transverse tubules or t-tubules. The transverse
tubules are found between sarcomeres at the border of the A and I lines. A triad is composed of a transverse tubule flanked on both sides by the sarcoplasmic reticulum. The tight coordination of this structure is the key to the ability of skeletal muscle to contract rapidly.

Skeletal muscle contraction is controlled by motor neurons. Each motor neuron contains multiple axons that control a group of muscle fibers termed a motor unit. Larger motor units are generally activated for powerful movements, while smaller motor units are activated for precision movements. When the motor neuron fires, acetylcholine is released from the synaptic bouton, which is linked closely to the endplate of the skeletal muscle. Acetylcholine crosses the cleft and binds to the acetylcholine receptor on the muscle fiber, which opens the receptor channel and depolarizes the membrane. This initial depolarization at the endplate causes sodium channels to open (mainly Nav1.4 in skeletal muscle), propagating a wave of depolarization which spreads over the surface of the fiber and down the t-tubules into the center of the fiber. Depolarization of the t-tubules causes a conformational change in the L-type calcium channel, which physically interacts with the SR calcium release channel, the ryanodine receptor and causes it to open. The opening of the ryanodine receptor allows calcium to flood into the cell and instigate muscle contraction. Calcium is then pumped back into the sarcoplasmic reticulum by the sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA) to allow another contraction cycle to initiate.

The muscle contraction described above consists of a contraction where the fiber is depolarized by a single action potential and then is allowed to relax. Because an action potential lasts only 5 ms while the calcium transient triggered by an action potential lasts around 50 ms, this means that multiple action potentials can be fired before a fiber can relax. Increasing stimulation frequency causes the calcium transients to fuse and increases the strength of
contraction. By this means of repeatedly firing action potentials, a muscle’s contraction can be sustained and increased to meet the demands of higher loads.

The ability of muscle to perform stable or dynamic movements is also a function of muscle fiber type. Skeletal muscle in mammals is mainly defined by slow twitch type I fibers, fast twitch oxidative type IIa fibers, and fast twitch glycolytic type IIx fibers (254). The slow twitch and type IIa fibers have a large capillary supply and rich mitochondrial content and thus have greater endurance than the type IIx fibers. However, the type IIx fibers typically have greater force production than either the IIa or the type I fibers. These fiber types have been defined by antibody staining to the myosin ATPase subunit that typifies each fiber type or by ATPase mediated staining which tends to correlate with antibody staining. The soleus muscle is a posture muscle and contains mainly type I muscle fibers in humans, while the quadriceps will contain a greater proportion of type II fibers as it is required for movement.

The fundamental mechanism by which muscle contraction is known to occur is called the sliding filament theory (254). This theory states that when muscle contraction is activated, the thin filament and thick filament slide along one another by myosin processively pulling and releasing an actin molecule in the thin filament. Myosin is only able to bind to actin after the inhibition of tropomyosin is removed via calcium’s interaction with troponin. Myosin then binds to actin with an ATP molecule already bound. In this state, myosin is only weakly bound to the actin filament (2). Then myosin hydrolyzes the ATP and through a molecular movement called the power stroke is able to undergo a large conformational change that moves the actin filament. After ATP is hydrolyzed, myosin becomes tightly bound. Pi and ADP then sequentially leave the ATP binding site and myosin becomes very tightly bound to actin. This state is termed the rigor state and is the cause of rigor mortis, which is the rigid state that an organism assumes.
shortly following death. When ATP binds the myosin molecule releases actin, returns to the ATP bound state and binds another actin molecule repeating the cycle. Understanding the fundamental biology of muscle contraction and organization is critical to understanding the disruptions that lead to weakness in disease states.

**Introduction to Duchenne Muscular Dystrophy**

Muscular dystrophies are diseases characterized by progressive loss of muscle tissue leading to weakness and muscle wasting. This is in contrast to myopathies, which are primarily characterized by ionic dysfunction of the muscle, which causes difficulties with muscle contraction or relaxation. The most common form of muscular dystrophy is Duchenne Muscular Dystrophy (DMD), which occurs in 1 in 5000 live male births (3, 4). Duchenne Muscular Dystrophy can be diagnosed within the first year of life through delayed achievement of developmental milestones, especially rolling over, crawling, and walking; however, this disease is still most commonly diagnosed in the toddler years (average age 4.7-5.7) where children are unable to keep up with their peers during play (5-9). These children are often mistaken for having increased muscle development as their muscles often appear to be hypertrophied and are hard to the touch. One manifestation of this is the so-called “woody calf,” which represents an inflammatory and fibrotic prodromal phase of the disease. As the hypertrophy present at this phase of the disease does not represent true muscle hypertrophy, it is often called pseudohypertrophy. At about the same time as pseudohypertrophy has developed, the patients have to push on their hips with their hands in order to stand up. This is called Gowers’ sign, and remains an excellent screen to detect muscle weakness in the hip girdle. Proximal muscle of the
hips, torso, and shoulders are affected first followed by involvement of more distal muscle groups.

The pseudohypertrophic phase of the disease eventually gives way to frank atrophy of the limb musculature as the muscles’ ability to regenerate itself is exhausted. This occurs around 10 years of age with the functional consequence of loss of ambulation. Some skeletal muscle groups appear to be largely spared from the disease including the laryngeal and extraocular muscles (10, 11). Additionally, Type I, also known as slow oxidative muscle fibers, tend to be better preserved than Type IIb or fast glycolytic muscle fibers which are nearly completely lost (12). The mechanism behind the differential susceptibility of different muscle types to disease is not entirely known but has been hypothesized to be due to alterations in the calcium release, buffering, and reuptake processes within these muscles.

Heart failure or respiratory failure (diaphragm) ultimately leads to the death of the patient (13, 14). The heart and diaphragm actually both endure the disease remarkably well given the continuous contraction cycle exposure imposed upon both of these muscles. Ventilator support is most commonly instituted in the late teens, which typically occurs years after the ability to ambulate is lost (15). Respiratory failure leading to pneumonia was the number one cause of death in DMD up until the last two decades. Advances in respiratory therapy including non-invasive ventilation (NIV) and mechanically assisted cough (MAC) have dramatically improved survival with numerous cohorts now surviving into their middle 30s (16, 17).

In recent years, cardiac failure has supplanted respiratory failure as the number one cause of death in DMD. Currently, 100% of DMD patients will progress to decompensated dilated cardiomyopathy, and there has been very little progress toward slowing the progression of the cardiac disease. The recent implantation of the HeartMateII and HeartWare Left Ventricular
Assist Device in DMD represents a novel therapeutic avenue that may be capable of preserving cardiac function for a greater period of time, much as advanced ventilator support has decreased deaths due to respiratory failure (18). A great degree of progress has been made in extending the lifespan in DMD, but further work is still necessary to better understand DMD heart failure to more effectively prevent it.

The recent breakthroughs in cardiac and respiratory treatment seem certain to prolong lifespan into at least the third decade and potentially longer in the near future. With the prolongation of life, improving the quality of life is the next frontier. Prolonging skeletal muscle function with minimal side effects is the ultimate goal. The only evidence based therapy for DMD is corticosteroids. Corticosteroids are believed to control the disease mostly through inhibition of the immune response, which exacerbates muscle damage in DMD. They may also exert protective effects on the muscle itself (19). Long term corticosteroid therapy has been associated with an array of benefits including a 2-5 year delay in loss of ambulation, improved pulmonary function, and improved cardiac function (20). Interestingly, the typical common side effects of steroid use including depressed mood, psychosis, hyperglycemia, insulin resistance, diabetes mellitus, cataracts, colitis, hypertension, hypothyroidism, buffalo hump, and retinopathy are not typically observed in DMD patients. Decreased height, obesity, and osteoporosis were observed as the primary side effects of corticosteroid use in DMD (20). Anecdotal evidence suggests that starting high dose steroids before age 3 can further prolong ambulation, but prospective clinical trials need to be performed before that can become the standard of care (21). Additional skeletal muscle therapies include surgery to decrease the painful scoliosis that occurs due to muscle weakness and use of orthoses to help prevent contractures (13). While prednisone
therapy has improved the course of disease, further therapies will be needed to further extend ambulation and improve quality of life in patients with DMD.

**Limb Girdle Muscular Dystrophy (LGMD)**

DMD is caused by the mutation of dystrophin which causes complete loss of dystrophin along with loss of many proteins in the dystrophin associated glycoprotein complex (DGC) which is made up of a large number of integral membrane glycoproteins (22). Dystrophin itself is a peripheral membrane protein, which is linked to the membrane through an interaction with the protein dystroglycan. Dystroglycan interacts with the sarcoglycan complex of α, β, γ, and δ sarcoglycan and with a series of extracellular proteins that ultimately connect to laminin in the basement membrane. The sarcoglycans are thought to have a signaling function and to also stabilize the membrane through their interactions with dystroglycan. Mutation of many of the proteins in the dystrophin glycoprotein complex has been linked to muscular dystrophy. Of these proteins, the δ sarcoglycan mutant is of particular importance to this work. Because the mdx mouse, which contains a loss of function mutation to dystrophin modeling DMD develops only mild pathology, our lab extensively utilizes the Sgcd-/- mouse which is deficient for the δ-

**FIGURE 3** – Illustrated model of the dystrophin glycoprotein complex. SS is sarcospan, Syn is syntrophin, nNOS is neuronal nitric oxide synthase, DGα is alpha dystroglycan, DGβ is beta dystroglycan, DB is dystrobrevin, α is alpha sarcoglycan, β is beta sarcoglycan, δ is delta sarcoglycan, γ is gamma sarcoglycan.
sarcoglycan protein. This mouse develops progressive pathology and fulminant necrosis which is more reminiscent of DMD. Both *mdx* and *Sgcd-/-* mice sustain loss of membrane expression of the other proteins of the DGC.

Another form of muscular dystrophy was also studied in this thesis which is not related to membrane fragility, but rather to membrane repair. *Myoshi’s Myopathy* or dysferlinopathy, as the name implies, are caused by mutations of dysferlin, a protein involved in membrane fusion and repair. In its absence, plasma membrane injury induced by a laser took longer to heal (23). The disease of *Myoshi’s myopathy* is characterized by initial weakness of distal musculature that eventually spreads proximally. Most commonly this disease arises in the teen years to the early 20s and while generally slower progressing than other forms of MD still leads to loss of ambulation by 60 years of age. In this case, the mechanism of diminished membrane repair may lead to a later disease onset due to the necessity for wear and tear to accumulate whereas the primary membrane defect in DMD and LGMD leads to early fulminant disease.

**Molecular Pathophysiology of Muscular Dystrophy**

The DMD locus was precisely located on the X chromosome in the early 1980s through a huge international effort (24-27). The cDNA expressed from this locus was later cloned and subsequently an antibody was raised to the protein dystrophin (28, 29). The crucial early development of an antibody to dystrophin, led to the discovery that the *mdx* mouse shared the same loss of function mutation as boys with DMD (28). It also led to the discovery that the protein dystrophin was a membrane associated protein that was localized to the triads; a critical region in the regulation of excitation contraction coupling where the t-tubules contact the SR (30-32).
The production of a dystrophin specific antibody also allowed scientists to find proteins that interacted with dystrophin. By enriching membrane fractions, dystrophin was determined to be a peripheral membrane protein tightly associated with at least four integral membrane proteins (33-36). Dystrophin’s interaction with membrane proteins was further characterized and the group of proteins with which it interacts is now called the dystrophin glycoprotein complex (37). Expression of these associated sarcoglycan proteins was found to be lost in the *mdx* mouse and DMD patients (35, 37-39). This suggested that dystrophin was necessary to stabilize the entire membrane protein complex. Further mapping of interaction with dystrophin went on to show that the N-terminus of dystrophin was bound to cytosolic actin. The dystrophin glycoprotein complex also bound to laminin in the extracellular matrix (40-43). Thus, dystrophin was shown to act as a link between the cytoskeleton and the extracellular matrix. The localization of dystrophin to both calcium release zones and as a bridge between the ECM and the cytoskeleton led to two opposing views of the pathophysiology of DMD. The membrane hypothesis held that dystrophin must be serving to strengthen the plasma membrane against contraction, while the calcium hypothesis held that disturbances in calcium handling led to elevation of cytosolic calcium and the induction of downstream necrotic mechanisms. These competing theories will be discussed in the following sections.

**The membrane hypothesis of muscular dystrophy**

The membrane hypothesis of muscular dystrophy emerged as a logical synthesis of much of the early data gathered about DMD. One crucial early observation was that muscle enzyme creatine kinase was increased in the serum of patients with muscular dystrophy (44, 45). Early EM studies of biopsies from DMD boys also observed muscle membrane breakage in muscle
fibers with degenerated mitochondria and sarcomeres, providing further evidence for membrane weakness as a cause of dystrophy (46). It was later shown that Evan’s blue dye, a dye that binds tightly to albumin, could infiltrate muscle cells (47). The infiltration was increased after contraction, further suggesting that membrane weakness contributed to muscle necrosis and weakness in muscular dystrophy (48). Dystrophin’s localization to a plasma membrane complex also fit well with an apparent structural instability of the plasma membrane. While these studies indirectly suggest that membrane weakness was the primary defect in mdx mice, further work was needed to directly measure the strength of the membrane in muscular dystrophy.

Direct measurement of a membrane defect in muscular dystrophy has been less convincing. It is possible to directly measure the tensile strength of a biological membrane by applying suction to a patch pipette and measuring the force at which the membrane breaks. Using this technique, it was shown by two groups that the force required to break the plasma membrane of myotubes and myofibers was similar between the mdx muscles and the control muscles (5, 49). This method was extended to vesicles obtained from mdx and control mice in case the previously discussed results were due to technical challenges caused by performing this technique in situ, but again it was shown the vesicles had similar mechanical properties (49). More modern data using atomic force microscopy to measure membrane elasticity have been mixed with one study finding a marked decrease in elasticity and the second study finding a marked increase (50-52). In contrast to studies attempting to measure physical properties of the membrane, studies of membrane fragility using hypotonic solutions have convincingly shown that mdx muscle fibers are more fragile than WT fibers (53). However, exposure to hypotonic solutions also causes calcium signaling to occur within a muscle fiber which may predispose it to necrosis (54), so this data does not definitively provide support for either the membrane or the
calcium hypothesis. Thus, while early observations pointed towards membrane defects, actual quantification of this deficit has proven difficult.

Regardless of the nature of a possible mechanical defect in the plasma membrane, restoration of membrane integrity through the DGC or other associated pathways has led to rescue of the disease phenotype. Importantly, membrane integrity is restored by dystrophin re-expression through transgenic methods, cell transplantation, and gene therapy, providing important proof of principle for future therapeutic strategies aimed at restoring dystrophin expression to the membrane (55). Interestingly, engineered dystrophin molecules including only the N and C terminus, but omitting many central domains of dystrophin are also effective at preventing muscle necrosis (56-58). The smaller dystrophin molecules can be packaged into viral vectors, providing a possible mechanism of gene delivery.

An alternative method of restoring membrane integrity is to increase the expression of the protein utrophin, a protein homologous to dystrophin (59, 60). Utrophin is initially expressed in the neonatal mouse and is replaced by dystrophin after birth. In the adult, utrophin localizes predominantly to the neuromuscular junction. In the mdx mouse models, transgenic overexpression of utrophin is able to substitute for dystrophin, restoring strength and decreasing membrane permeability (61). Other possible strategies include the use of small molecules that can upregulate utrophin expression (60). Another interesting strategy to restore membrane function utilizes overexpression of integrin to restore membrane structure through an alternative, dystrophin independent linkage pathway between the ECM and actin cytoskeleton (62). Overexpression of other proteins in the dystrophin glycoprotein complex, such as sarcospan and biglycan, have also been shown to restore muscle function presumably by overriding the dystrophin deficiency (63, 64). Pharmacologic stabilization of the membrane has recently
become possible through the use of the agent Polaxmer-188. This molecule decreased cardiomyocyte membrane permeability and rescued cardiac muscle pathology; however it had no effect on skeletal muscle (65, 66). Rescue of muscular dystrophy pathology by multiple membrane proteins suggests that membrane instability contributes to the pathophysiologic mechanism of muscular dystrophy.

In order to better engineer truncated dystrophin molecules for gene therapy, a greater understanding of the biophysical function of dystrophin was needed. While it was very quickly appreciated that dystrophin bound to both a membrane complex linked to the cytoskeleton and to the actin cytoskeleton, the mechanism by which dystrophin impacted the cytoskeleton was only recently elucidated. Recent work using time resolved phosphorescence anisotropy found that dystrophin is not merely a linkage between the cytoskeleton and the ECM, but that it can actually dampen the normal movements of actin, analogous to the actions of a shock absorber in a car (67, 68). Surprisingly, these studies found that micro-dystrophin was actually more similar biophysically to full length dystrophin than the longer protein mini-dystrophin (67). Further iterative optimization of truncated dystrophin molecules will be critical to maximizing the therapeutic benefit of future gene therapy efforts.

The membrane hypothesis has produced many potential therapies, suggesting the utility of this theory; however, the actual demonstration of membrane weakness remains elusive. Thus, it is possible that the rescues observed may not be through repair of membrane strength but another mechanism altogether. It is perhaps more likely that the membrane hypothesis and the calcium hypothesis are intertwined, as modulation of both pathways can ameliorate the dystrophic phenotype. It is also important to note that calcium overload itself, as occurs in TRPC3 Tg mice and Stim1 Tg mice, does not lead to increased membrane permeability but does
lead to muscle necrosis and weakness reminiscent of muscular dystrophy (69, 70). Thus, further work is necessary to understand the interaction between the membrane and calcium hypothesis of muscular dystrophy. The following section will discuss the calcium hypothesis in detail, as this hypothesis is most pertinent to the data presented in this thesis.

**The calcium hypothesis of muscular dystrophy**

The calcium hypothesis of muscular dystrophy states that calcium overload in dystrophic skeletal muscle causes necrosis through mitochondrial and sarcomeric destruction. This theory was first elucidated in its modern form in the *Lancet* in 1976 where Wrogeman, et al. suggested that a sarcolemmal defect leads to mitochondrial calcium overload, which subsequently leads to further ionic dysregulation and muscle contracture (71). The evidence that supports the steps of these pathways has increased exponentially in the past 10 years due to genetic manipulation of the mouse and will be discussed in detail in the following sections.

**Muscle Contraction**

The first evidence that calcium levels may be deranged in muscular dystrophy came from studies of muscle contraction in muscle fibers from boys with DMD. Muscle weakness is hallmark of DMD and whole muscle contractile properties have reproducibly shown weakened and slowed muscle contractions in DMD boys (72, 73). Measuring contraction in single muscle fibers from DMD patients and their carrier mothers showed that while only the DMD patients had muscle weakness, both the carriers and patients had slowing of muscle relaxation, suggesting that dystrophin deficiency causes an intrinsic defect in sarcoplasmic reticulum function (74). This prolongation of relaxation also suggested that a prolonged elevation of cytosolic calcium occurs in DMD. Studies of muscle from boys with DMD helped to establish some of the basic
tenants of dystrophic muscle pathophysiology, but a greater understanding was gained after the 
*mdx* mouse was found to carry the same mutation as human DMD.

The use of the *mdx* mouse model in later studies allowed more intensive investigation of 
contractile defects in muscular dystrophy. However unlike DMD, *mdx* mice have near normal 
life span and are not crippled by the disease (75). In fact whole muscle force may be greater in 
*mdx* mice than wild type (76). Reports of the deficit in specific force in the *mdx* mouse suggest 
only a modest force deficit of 10-20% (76-78). However, relaxation times are increased in *mdx* 
mice by 50-300% depending on the method and system used, showing that a deficit in relaxation, 
potentially leading to calcium overload is an evolutionarily conserved aspect malfunction of the 
DGC (79, 80). Early studies in the *mdx* mouse reproduced the defect in relaxation that was 
previously observed in DMD boys, but the force deficit in DMD boys was not reproduced. 
Future studies found other profound deficits in *mdx* mouse that may point the way toward 
mechanisms of muscle damage in humans.

Dramatic deficits in force production are present following eccentric contraction in the 
*mdx* mouse. An eccentric contraction is one where the muscle is lengthening during active 
contraction. Examples of this type of contraction occur when one lowers a heavy object, lowers 
one'self into a chair, or descends a flight of stairs. Force deficits from 40-70% occur in the *mdx* 
mouse following eccentric contractions while force drops from concentric (shortening) 
contractions are <5% (81-83). High levels of dye permeability are also observed in *mdx* muscle 
following eccentric contraction, but dye permeability is not observed in control muscle (84). 
Interestingly, the defect in eccentric contraction was specific to fast twitch *mdx* muscle, because 
the predominantly slow twitch soleus muscle displayed no force deficit following eccentric 
contraction (81). The protection of slow fibers has also been demonstrated in DMD boys where
it was found that almost no fast type II glycolytic fibers had survived and were instead replaced by slow Type I fibers and Type II oxidative fibers (85). These studies strongly suggest that eccentric contraction leads to muscle necrosis in the fast twitch muscle fibers of DMD patients and mdx mice.

Eccentric contraction induced force decrement is much greater in mdx mice than wild type mice in part due to increased muscle permeability and necrosis in mdx mice. The increased permeability suggests a mechanical deficit in the muscle membrane; however, the contractions also elicit large increases in intracellular calcium and sodium, which may in turn lead to necrosis and increased permeability (86, 87). Interestingly, the elevation of sodium and calcium and the damage incurred by eccentric contraction can be blocked by inhibitors of stretch activated channels such as streptomycin and gadolinium, suggesting that the ionic dysregulation and subsequent necrosis are due to the activity of stretch activated channels and not due to microtears in the plasma membrane (86, 88).

Sodium and calcium entry following stretch may also be increased due to a newly discovered signaling pathway called X-ROS. In skeletal muscle the X-ROS pathway is triggered by an aberrantly polymerized microtubule network that activates NADPH oxidase in the t-tubules and plasma membrane (89). NADPH oxidase activity and ROS production have also been shown to act in positive feedback loops; thus, inhibition of ROS production or calcium entry can prevent stretch induced damage (54). Depolymerization of microtubules by colchicine also inhibited reactive oxygen species production and excessive stretch activated channel activity in vitro and decreased contraction induced muscle damage in vivo (89). An alternative mechanism of stretch activated channel activity has also been described to be due to increased phosphorylation of TRPC1 through Src, suggesting another level of regulation to the X-ROS
pathway (90). The eccentric contraction mediated X-ROS pathway represents an area of multiple signaling nodal points but more in vivo testing is required to ensure that the mostly in vitro observations discussed above translate into the intact organism.

In addition to studies of muscle contraction in isolated muscles or muscle fibers, muscle function in mdx and wild type mice has also been studied in the intact organism, most commonly through forced treadmill running to exhaustion. Mdx mice show severe deficits in running time and distance relative to control mice (91-94). This deficit is further exacerbated by running the mice downhill, which increases muscle damage through eccentric contraction (94). Interestingly, the damage incurred does not appear to peak during the exercise session but rather increases over several hours following exercise, again arguing for an ongoing necrotic process, and not simply mechanical weakness as the mechanism of dystrophic pathology (91, 95).

**Action Potential**

The skeletal muscle action potential is a highly coordinated, extremely rapid event, lasting only 5ms from start to finish. As discussed previously, the action potential begins with Ach binding to the AchR causing opening of the AchR, leading to depolarization that is propogated down the t-tubules by Nav1.4, the skeletal muscle sodium channel. This depolarization is required for a conformational change in the L-type calcium channel that triggers excitation contraction coupling. The action potential can be repeated over 100 times per second, making relatively minute changes in action potential duration very important to skeletal muscle calcium handling.

While electrophysiology has long been used in the diagnosis of muscular dystrophy, measurement of the skeletal muscle action potential in muscular dystrophy has been very limited.
The initial study of \textit{mdx} and WT fibers found no significant difference between the groups (96). The second study performed on this topic included the \textit{mdx utrophin-/-} mice (DKO) which lack both dystrophin and utrophin (97). The DKO mice have a severe pathological course but are not routinely used due to difficulty breeding these mice. The DKO mice were discovered to have two populations of muscle fibers. One population had action potentials similar to wild type and the second population contained fibers with greatly prolonged decay of the action potential. These fibers also had much slower decay of the calcium transient. This could suggest that some of the dysfunction of calcium release and reuptake may have its origins in the generation of the action potential. It also highlights the concept that two populations of fibers with different calcium handling characteristics may exist within the dystrophic organism.

**Excitation Contraction Coupling**

Excitation contraction coupling is the process by which membrane depolarization leads to calcium release and muscle contraction. Previous studies of muscle fiber biopsies from boys with DMD demonstrated defects in muscle strength and relaxation. The importance of weakness to DMD is obvious, but relaxation defects are also important because they expose the cell to prolonged calcium overload. Recently, the calcium handling process has been dissected to better understand the defects in contraction and relaxation that were observed.

The invention of fluorescent calcium indicator dyes made it possible to ask whether weakness and muscle damage could be caused by defects in calcium handling. Initial studies by Head found that excitation contraction coupling was unchanged in \textit{mdx} mice compared with wild type controls (98). Others subsequently found that the decay phase of the calcium transient is more prolonged in \textit{mdx} muscle fibers consistent with delayed relaxation observed in intact muscle (92, 99). In the above studies, no alteration in the amplitude of the calcium transient was
noted, suggesting that a similar amount of calcium was released in dystrophic and wild type fibers. More recent studies, utilizing low affinity calcium indicator dyes, which more faithfully measure the calcium transient, along with sophisticated computer modeling to estimate calcium release, have shown that calcium release is decreased in *mdx* fibers (100). While calcium release typically occurs over tightly defined areas called triads in skeletal muscle, it was recently shown that calcium release occurs much more diffusely in *mdx* fibers (101). This is interesting because dystrophin localizes to the triad and may thus have a role in patterning calcium release.

Calcium release dysregulation in dystrophy is also evidenced by the presence of the calcium spark. The calcium spark signifies the opening of a group of ryanodine receptors and is the fundamental calcium release event in cardiac muscle; however, it is typically absent from wild type skeletal muscle. Thus, the presence of calcium sparks in dystrophic skeletal muscle suggests it may contribute to the disease process (102, 103). Calcium sparks are increased in muscular dystrophy in part through increased nitrosylation of the ryanodine receptor, which causes dissociation of the protein calstabin, leading to increased spontaneous opening (104, 105). The interaction of calstabin with the ryanodine receptor can be restored by the drug S107, (105) and treatment of *mdx* and Beta-sarcoglycan deficient mice with S107 decreased histopathology and increased muscle strength (106, 107). This suggests an intrinsic deficit in calcium release also contributes to disease progression in muscular dystrophy.

In addition to calcium release, calcium reuptake is also impaired in dystrophic mice. Decreased Sarcoplasmic Reticulum Calcium ATPase (SERCA) activity was initially observed in microsomal membrane preparations from *mdx* mice, dy2j/dy2j mice, and DMD boys (108-110). This result was later confirmed *in situ* in muscle fibers (79, 92). Recently, Goonasekera et. al. showed that the calcium reuptake deficit can be corrected by overexpression of the SERCA1.
The correction of calcium reuptake was also shown to greatly decrease dystrophic histopathology in both the *mdx* and the *Sgcd-/-* mouse model. Furthermore, adeno-associated virus delivery of SERCA2 was also able to diminish histopathology (92, 111). Because this vector is in trials for human heart failure, it may be translatable to the clinic sooner than other therapeutic strategies. Taken together, this work substantiates the supposition that the delayed relaxation observed in dystrophic muscle may be contributing to dystrophic pathology.

**Intracellular Calcium**

Intracellular calcium levels regulate the activity of numerous cellular processes. If intracellular calcium becomes too great, mitochondrial dysfunction and proteolytic digestion of the muscle will occur. Early studies of calcium in muscular dystrophy performed in the 1970s analyzed biopsy specimens from boys with DMD. Three techniques available at the time, X-Ray fluorescence, histochemical staining, and atomic absorption spectrophotometry, demonstrated an increase in muscle calcium relative to controls (112-114). Histochemical techniques showed that 4.83 percent of muscle fibers from DMD boys were von Kossa positive, while only 0.21 percent of control fibers were positive. X-Ray fluorescence and atomic absorption spectroscopy found whole muscle calcium content was increased, but only crude correlations to intracellular versus extracellular calcium could be made. Thus, new techniques were needed to quantify and localize the increased calcium observed by these early studies.

In the late 1980s and 1990s, measurements of intracellular calcium in WT and dystrophic mice were made utilizing newly available calcium sensitive dyes. The initial studies of Turner et. al. using the dye Fura-2 injected into mechanically isolated skeletal muscle fibers found that resting intracellular calcium in wild type fibers was 40nM +/- 2.8 and 92nM +/- 9.8 in dystrophic fibers (115). Gaillly et. al. found a large difference in the affinity of Fura-2 for calcium within
wild type and *mdx* muscle fibers. This difference in calibration of the measurements made the resting calcium equal between wild type and *mdx* fibers (116). Alternatively, Head found there were two populations of *mdx* fibers (98). The first population had resting membrane potentials and resting intracellular calcium identical to wild type, while the second population had depolarized membrane potentials and increased intracellular calcium. Another study utilizing calcium sensitive dye, Indo-1, also came to the conclusion that resting calcium was not different between wild type and *mdx* mice (117). The study of Turner et. al. differed from the other studies, in that Turner, et. al. used mechanical dissection to isolate *mdx* muscle fibers while the others used collagenase digestion. It was suggested by Head, that collagenase digestion may act as a form of selection by killing the weaker depolarized fibers. Gailly also used passive loading of the Fura-2AM form which is de-esterified intracellularly, while Turner injected the Fura-2 quaternary amine. Passive loading may lead to overloading of cells with dye and to dye penetration into intracellular organelles. It is important to recognize that Fura-2 and Indo-1 are high affinity calcium buffers, and high dye concentrations can obliterate small differences in calcium. More studies and alternative techniques were needed to clarify the cellular calcium levels of *mdx* and wild type mice.

Ca-sensitive microelectrodes were recently applied to myotubes from *mdx* mice. In these experiments, Altamirano, et. al. showed that intracellular calcium was increased to 308nM +/- 6nM in *mdx* myotubes compared to 113nM +/-2nM in wild type myotubes (118). While these concentrations were much greater than the concentrations measured with calcium sensitive dyes, the buffering may cause dyes to underestimate intracellular calcium. This method also allows for *in vivo* measurement of intracellular calcium and sodium. Using this technique, resting
calcium was measured to be 315nM+/8nM in *mdx* mice and 112 nM +/- 2nM in wild type mice (119).

Alterations in localization of calcium have also recently been explored in *mdx* mice. The first suggestion that calcium microdomains may be altered in muscular dystrophy was provided by Turner who showed that rapidly elevating extracellular calcium caused the intracellular calcium to increase rapidly in *mdx* fibers but not in wild type fibers (120). Increased local calcium concentrations were more definitively shown by Mallouk et. al., who used calcium activated potassium channel current to detect increased subsarcolemmal calcium concentrations in *mdx* mice (121). Recently, this result was confirmed using a membrane localized calcium sensitive dye FFP-18 that showed bulk cytosolic calcium was similar between *mdx* and wild type mice, whereas subsarcolemmal calcium concentrations were elevated in *mdx* mice (122). Thus, while the story on resting calcium is still evolving, new methods suggest that intracellular calcium may be increased in *mdx* mice, especially in the subsarcolemmal region.

**Store Operated Calcium Entry and Stretch Activated Calcium Entry**

In the 1976 Wrongman and Pena paper, increased calcium entry through the plasmalemma was identified as the first step of the pathological process; however, the origins of this calcium entry have remained elusive for many years. The first evidence of calcium entry through the plasma membrane came in 1988 when Turner et. al. found that increasing extracellular calcium dramatically increased calcium in *mdx* muscle fibers but not in wild type muscle fibers. At that time, the mechanism of calcium entry was completely unknown. This initial observation led others to measure calcium currents in dystrophic muscle. These measurements showed a large increase in voltage independent calcium current in *mdx* myotubes.
This form of calcium entry was found to be activated by hypo-osmotic stress (99, 124, 125). This current was sensitive to lanthanum and gadolinium, suggesting it utilized the same channels as previously described stretch activated currents (124, 125). Vanderbrouk et. al. hypothesized that this current was due to TRPC proteins and they were able to show that an antisense oligonucleotide which targeted the entire TRPC family of proteins was able to silence the voltage independent calcium current (126).

The seven TRPC proteins form heterotetrameric calcium entry channels that open in response to stretch, decreased sarcoplasmic reticulum calcium content, and the molecule diacylglycerol (127-129). In skeletal muscle, overexpression of TRPC3 also increases calcium entry in response to store depletion and is sufficient to induce skeletal muscle pathology in vivo (70). Conversely, overexpression of dnTRPC6 ameliorates dystrophic pathology in Sgcd-/- and mdx mice, and it restores store operated calcium entry in mdx and Sgcd-/- mice to wild type levels (70). Thus, store operated calcium entry is both a necessary and sufficient component to development of the dystrophic phenotype.

The store operated calcium entry (SOCE) process functions to refill the calcium store upon its depletion and as a pathway for calcium signaling. The predominant regulators of this pathway are the SR calcium sensor, Stim proteins, and the plasma membrane calcium channel, Orai proteins. The TRPC proteins are accessory proteins to this process and may augment its function. Recently knockdown of Orai1 with shRNA was shown to decrease store operated calcium entry in mdx mice and to decrease muscle pathology (130). Other work using skeletal muscle transgenic strategies has shown that Stim1 Tg is highly pathogenic in both cardiac and skeletal muscle (69) and personal communication with Sanjeewa Goonasekera). The relevance of these in vivo findings has also come into question by physiologic studies, which suggest that
calcium entry through the SOCE pathway is compensatory and not pathological (131). Despite the physiologic studies, small molecule inhibitors to proteins in the SOCE pathway represent an attractive area for future translational investigation.

Stretch activated calcium entry may also be involved in the development of dystrophic pathology. The dramatic damage caused by eccentric contraction, which can be inhibited by gadolinium is excellent evidence for the relevance of this pathway to the pathogenesis of muscular dystrophy. While the TRPC proteins, especially TRPC1 have been shown to mediate stretch activated calcium entry in other tissues, more evidence supports the role of the TRPV proteins in stretch activated calcium entry in dystrophic muscle. The protein TRPV2, is a voltage independent calcium channel that has also been shown to be involved in the pathology of muscular dystrophy in the mdx mouse and Sgcd-/ hamster through development of dnTRV2 transgenic and viral delivery mechanisms (132). TRV2-/ mice also exhibit less pathology in the mdx background, suggesting that the disease is caused by TRV2, not through the dominant negative effect of the transgene on other ion channels (133). X-ROS was previously discussed but is also an exciting area of research into the stretch activation of calcium channels (89).

Finally, the antibiotic drug streptomycin has also been shown to inhibit stretch activated calcium channel activity and to ameliorate the mdx phenotype (86, 134). Unfortunately, chronic use of this drug adversely affects the heart and diaphragm, likely through inhibition of mitochondrial ribosomal activity (135). More specific inhibitors targeting stretch activated channels are now being developed that will allow the translation of the growing body of fundamental research into clinical practice.

The final way calcium channels are known to be activated in skeletal muscle is through receptor activation mechanism, known as receptor operated calcium entry (ROCE). Calcium
entry through ATP gated channels has been the most studied pathway in this category. ATP is released from inflammatory cells within dystrophic muscle and may contribute to dystrophic pathology. Pharmacological inhibition of the P2X7 ATP activated channel has been shown to decrease dystrophic histopathology (136). A pathway that has been much less studied is DAG mediated opening of calcium channels through PLA2 signaling (137). While manipulation of this pathway has not been directly tested in vivo, PLA2 signaling has been implicated in the development of the muscle necrosis induced by cardiotoxin (138). Because the calcium entry caused by PLA2 agonists is profound, the in vivo relevance of this pathway requires testing.

**Calcium mediated protease activity**

Calcium activated proteases called calpains are critical to muscle development and homeostasis. In the absence of calpain-3, the triad fails to form appropriately, leading to a form of limb girdle muscular dystrophy (115). However, increased calpain activity can also exacerbate pathology in muscular dystrophy. Calpain expression and activity are increased in muscle from mdx mice (139). Increased calpain activity has also been demonstrated in situ in mdx muscle fibers, showing that the increased activity present in the lysates was not due to immune infiltrate (140). To test the involvement of calpains in the disease process, Spencer et al. overexpressed the protein calpastatin, an endogenous calpain inhibitor, in the mdx mouse (141). Overexpression of calpastatin was sufficient to ameliorate dystrophic pathology. Interestingly, calpastatin overexpressing mice had less necrotic lesions in sections, but membrane permeability to dye was not changed. A subsequent study using leupeptin, a protease inhibitor with some specificity to calpains, found less pathology in the treatment group (142). Recently, Briguet et al. repeated overexpression of calpastatin in the mdx mouse and failed to appreciate a difference in muscle pathology; however, when they inhibited both calpains and the
20S proteasome with SNT198438, they were able to rescue the dystrophic phenotype (143). They also found that the 20S protease system activity was induced by increased intracellular calcium. While the most recent study questions the source of calcium dependent protease activity, the overall conclusion of all studies to date suggest that calcium potently activates proteolytic activity in dystrophic muscle, and this activity likely contributes to necrosis in muscular dystrophy.

**Mitochondrial Permeability Transition Pore Opening**

Mitochondrial permeability transition pore (MPTP) opening results in depolarization and swelling of the mitochondria leading to loss of energy production. While this process can potentially preserve mitochondria by allowing calcium efflux to occur, loss of energy production can result in loss of plasma membrane ionic regulation and subsequent necrosis. Thus, precise regulation of the pore is crucial. The mitochondrial permeability transition pore (MPTP) is a multiprotein complex found on the inner membrane of mitochondria. The identities of the specific proteins that compose this complex remain controversial; however, its opening is regulated by the prolyl isomerase cyclophilin D (144, 145). In particular, cyclophilin D appears to sensitize the pore to opening in the presence of elevated levels of intracellular calcium.

Because cyclophilin D (CypD) sensitizes cells to MPTP, mice lacking CypD were crossed into the *mdx* and *Sgcd-/-* backgrounds to determine if CypD deletion desensitized dystrophic mitochondrial to MPTP opening. The mitochondria from *Sgcd-/-* mice are swollen at baseline suggesting that MPTP opening is occurring (146). The deletion of *Ppif* decreased mitochondrial swelling in *Sgcd-/-* mice. Importantly, deletion of CypD led to histologic rescue of the dystrophic phenotype and restoration of force in the *Sgcd-/-* background. Millay et. al. also showed that inhibition of cyclophilin D by the specific inhibitor Debio-025 ameliorated
dystrophic pathology. Subsequent studies found that Debio-025 decreased necrotic pathology in the *mdx* mouse (147, 148). It was also shown that Debio-025 treatment restored functional properties to dystrophic muscle including improving relaxation and resistance to contractile damage (148). The inhibition of MPTP by pharmacologic and genetic mechanisms was later shown to be protective in Ulrich congenital muscular dystrophy as well (149, 150). These results suggest that MPTP opening plays a prominent role of dystrophic pathology, likely as a downstream consequence of calcium overload.

**Na Homeostasis**

The gradient of sodium ions across the plasma membrane is the basis for excitability and active transport. The energy from this gradient drives the active transport of metabolites critical to life. It also allows nerve, muscle, and cardiac cells to fire action potentials by quickly opening channels that allow a small amount of sodium ions across the membrane. The sodium gradient also provides energy to balance large changes in other ions, through exchangers such as the sodium calcium exchanger, the sodium-potassium-calcium exchanger, and the sodium hydrogen exchanger. In living organisms, this gradient never reaches equilibrium but it does achieve a steady state balance of sodium influx and sodium pumping across the membrane by the Na,K-ATPase. The result of this balance is the intracellular sodium concentration. The lower the intracellular sodium concentration, the greater the gradient and the more potential energy is available for pumping and exchanging. Increased intracellular sodium levels can actually cause exchangers to reverse and can cause cellular swelling to osmotic effects. The following paragraphs will describe what is currently known about the regulation of sodium transport in muscular dystrophy.
The first study that measured intracellular sodium in \textit{mdx} mice found a dramatic elevation of resting sodium levels from $13 \pm 3$ mM to $24 \pm 2$ mM in the gastrocnemius and from $13.0 \pm 0.3$ mM to $23.5 \pm 0.7$ mM in the diaphragm (151). Resting sodium levels of 11.5mM in wild type and 22.5mM in \textit{mdx} mice were subsequently measured using a dye based method, suggesting that the above results were accurate (152). Intracellular sodium measurement has also been extended to DMD patients using Sodium 23 MRI. Using this technique, Weber, et al. estimated the intracellular sodium in control subjects was 25.4mM sodium, while subjects with DMD patients had intracellular sodium of 38.0 mM, suggesting that sodium overload may be a larger component of the disease process in humans (153, 154). The high resting sodium level could lead to cellular edema or increased calcium entry through the reversal of the sodium calcium exchanger. Further work was necessary to find the source of this increase.

The increase in intracellular sodium observed in the \textit{mdx} mouse requires that there be either increased influx or decreased efflux at steady state relative to the wild type situation. Interestingly, Na, K ATPase activity was found to be increased in the \textit{mdx} muscle lysates (155). This was likely due to overexpression of the ouabain sensitive, \textit{a}2 Na,K-ATPase (156). There are several explanations to potentially reconcile the observed simultaneous increase in sodium potassium ATPase activity and increased intracellular sodium levels. One explanation would be that sodium influx has increased to an even greater extent than Na, K-ATPase activity. It is also possible that unlike these studies where ATP was supplied in excess in the buffer, ATP is actually a limiting reactant \textit{in vivo} due to mitochondrial dysfunction, which would slow the \textit{in vivo} pumping rate despite increased expression of the pump.
Investigations into sodium influx suggest multiple sodium influx pathways are activated in muscular dystrophy. Using the sodium sensitive dye, SBFI, resting sodium influx was shown to be equivalent between WT and *mdx* mice (157). Sodium influx was found to be increased when muscle fibers were electrically stimulated (158). It was also shown that sodium influx occurred during lengthening or eccentric contraction but not during isometric contractions (87). This is not surprising because the channels that allow calcium entry during eccentric contraction are non-selective cation channels. Overall, much more work will be required to understand basic facts about how sodium balance is regulated in muscular dystrophy.

The role of sodium has also been investigated in muscular dystrophy in vivo. The first study investigated the premise that the driving force for sodium could also be increased by increasing extracellular sodium. They tested their hypothesis by infusing hypertonic sodium into the mice every day to increase extracellular sodium (159). They found that a diet containing 12% sodium chloride decreased dystrophic histopathology and speculated that this may be due to increased forward mode sodium calcium exchange. A second study took the approach of inhibition of the sodium hydrogen exchanger to decrease sodium influx (160). They found intracellular pH was actually elevated in the BIO14.6 hamster, which they attributed to constitutive activity of the sodium hydrogen exchanger. They thought this would contribute to pathology through calcium overload through reverse mode sodium calcium exchange. When they blocked the sodium hydrogen exchanger with cariporide they found that intracellular sodium and calcium were decreased, mice were protected from stretch induced damage, and the general histopathology was improved.
Sodium Calcium Exchange

Sodium calcium exchange and the pumping of calcium by the plasma membrane calcium ATPase (PMCA) are the only two methods of cellular calcium removal currently known. While the pump always moves calcium from the intracellular space to the extracellular space at the cost of ATP hydrolysis, the sodium calcium exchanger can mediate calcium influx or efflux from the cell depending on the gradients of sodium and calcium across the plasma membrane and the membrane potential. When the cell membrane is hyperpolarized and sodium levels are low the exchanger mediates calcium efflux; however, when intracellular sodium levels rise and the membrane potential is depolarized the exchanger mediates calcium entry. The main role of the exchanger appears to be calcium efflux, especially when the intracellular calcium concentration is high; however, under certain circumstances such as treatment with the sodium pump inhibitor digoxin, the exchanger mediates additional calcium influx, which can enhance contractility. Having only a calcium pump on the plasma membrane would seem to be a much simpler and more efficient system for the cell to utilize; however, the exchanger has a 10-50 fold greater turnover rate than the pump. This means that the exchanger has the ability to play a much greater role in regulating calcium homeostasis in excitable cells like neurons, cardiomyocytes, and skeletal muscle fibers where large calcium movements are common. The next section will briefly outline what has been learned about the exchange in other systems and attempt to relate that knowledge to muscular dystrophy.

Historical Perspective

The first observation of reverse mode sodium calcium exchange was published in 1921, only 40 years after calcium was discovered to mediate muscle contraction (161). In these
experiments, Daly and Clark, found that depleting extracellular sodium led to an increase in contractile force in cardiac tissue (162). They realized that this resembled the greater contractility observed when cardiac tissue was treated with strophthandin, a sodium potassium ATPase inhibitor, but did not articulate how these two processes were linked. These and other experiments led to the concept that sodium and calcium competed for entry through the plasma membrane.

In the late 1960s, three labs working in three different models discovered that the competition that previous investigators had seen was actually the exchange of sodium for calcium. The concept of sodium mediated counter-transport systems was first discovered in the 1950s in relation to solutes, but it had never been extended to modulation of ionic homeostasis. Reuter’s lab found that the exchange mechanism they helped discover was actually the predominant mechanism of calcium efflux in cardiac myocytes (163, 164). This is still believed to be the primary physiologic role of NCX1 though recent genetic studies show that while it does predominate, it is not required for normal functionality of cardiac tissue (204). The labs of Baker and Hodgkin found that exchange was possible both in forward and reverse modes depending on the gradient of sodium across the membrane (165, 166). Many questions still remained to be solved in the next decades including the coupling rate of the exchanger and unraveling the complex physiologic roles that the exchanger played.

In the 1980s, an exchange ratio of three sodium ions for one calcium ion was established for sodium calcium exchange across a host of diverse systems (167-171). The kinetic and equilibrium parameters defined during the 1970s and 1980s also helped to elucidate the physiologic roles of NCX1 and the “rules” of sodium calcium exchange. A fundamental rule was that intracellular calcium could allosterically activate the exchanger regardless whether the
exchanger acted in influx or efflux mode (172, 173). Specifically the K0.5 for intracellular calcium activation of NCX1 was found to be 1uM (174). This suggested that NCX1 was most active at high values of calcium, with the majority of exchangers inactivated by low resting intracellular calcium concentrations. Interestingly, allosteric activation of the exchanger is absent in the rat and mouse sodium calcium exchangers but is present in the exchangers of higher mammals. Sodium was discovered to biphasically regulate calcium efflux, with low intracellular sodium levels activating the exchanger while high levels inhibited exchange (175). ATP also likely regulates the exchanger through phosphorylation, increasing its activity (175, 176). All allosteric regulation of the exchanger appears to be linked to the intracellular loop, as intracellular chymotrypsin digestion and molecular biology mediated deletion of the intracellular loop removed allosteric regulation of the exchanger (177-181).

The SLC8a1 gene

In 1988, Philipson isolated the sodium calcium exchanger from sarcolemmal vesicles (182). The isolation of NCX1 allowed the raising of NCX1 specific polyclonal antibodies and the cloning of the NCX1 gene (183). They then expressed NCX1 in frog oocytes and showed that the expression of the gene they cloned was responsible for sodium calcium exchange (183). The antibody recognized three protein fragments on Western blot with 70, 120, and 160 KDa sizes. The smallest band was subsequently shown to be a cleaved form of

![Figure 4](image.png)
NCX1 while the larger bands represent the full length protein in different conformations. The canine NCX1 mRNA contains 2910 coding bases and encodes a protein of 970 amino acids (183). The cloning of NCX1 allowed the tools of molecular biology to be applied toward the dissection of NCX1 structure and function.

Using molecular biology, the topology of NCX1 was then mapped and it was found that NCX1 consists of three major domains. The N-terminal domain consists of 5 transmembrane segments, followed by a large cytoplasmic regulatory domain, and then 6 more C-terminal transmembrane segments (183). Using the Ca45 overlay method, a high affinity calcium binding region was found that corresponded to the intracellular loop spanning amino acids 371 to 508 (184). Finer mapping studies have shown that there are two high affinity calcium binding sites in the intracellular loop 446-448 and 498-500 (180). Studies simulating chymotrypsin digestion showed that a NCX1 protein lacking this loop from AA240 to 680 led to generation of a fully functional protein exchanger and lacked allosteric regulation (181). Downstream of the crucial calcium binding segments, amino acids 680 to 685 are also required for allosteric activation of NCX1 (185). Another crucial structural region was also identified and termed the X-inhibitory peptide (186, 187). The XIP is a 20 amino acid region located at the amino terminus of the intracellular loop and overexpression of this region potently inhibits exchanger function (186).

The Slc8a1 gene, which encodes the NCX1 protein, is on chromosome 2 in humans and 17 in mice and spans 365 and 400kb of DNA respectively. The first exon of this massive gene is composed of one of two obligate and mutually exclusive exons termed A and B. The A isoform is expressed in the heart and other excitable tissues, while the B isoform is expressed in the kidney and non-excitable tissues. Exons C, D, E, and F are cassette exons, which can be alternatively spliced to form NCX1 isoforms with altered properties and tissue distributions.
Surprisingly, some isoforms of NCX1 are more different in terms of activity from the cardiac isoform (NCX1.1) than NCX2 or NCX3 which are expressed from separate loci (188). While this tremendous variability is thought to allow tissue specific functionality and adaptability to the utilization of NCX1 by cells, the differing properties and regulation of alternative splicing remain largely unexplored. There are 32 possible isoforms, of which 10 are expressed in skeletal muscle. Interestingly, skeletal muscle is believed to be the only tissues, with differential expression of NCX1 isoforms during development. Neonatal mice express NCX1.2, NCX1.3, NCX1.7, and NCX1.10, with expression changing to NCX1.1 (cardiac isoform), NCX1.2, NCX1.3, and NCX1.9 in the adult (189). Alternative splicing of NCX1 likely represents an important mechanism to provide tissue specific modulation of NCX1 function, but the details of this process remain largely unexplored.

**NCX1 Regulation**

NCX1 is regulated primarily by sodium and calcium gradients, as well as, the membrane potential. NCX1’s activity rapidly balances large perturbations in any of these parameters by utilizing energy stored in the gradients of the other two parameters. NCX1 can act in one of two modes of transport: forward and reverse mode. In forward mode, NCX1 transports three sodium ions into the cell while extruding one calcium ion. This charge movement generates a net positive charge transport into the cell, which is depolarizing. Forward mode transport is favored by high intracellular calcium concentrations, low intracellular sodium concentrations, and a hyperpolarized membrane potential. These conditions would predominate during prolonged muscle activity such as aerobic exercise. In reverse mode sodium calcium exchange, one calcium ion enters the cell in exchange for the extrusion of three sodium ions. This movement of charge repolarizes the cell. Reverse mode sodium calcium exchange is favored by high levels of
intracellular sodium, low levels of intracellular calcium, and a depolarized membrane potential. These conditions would occur in a wild type muscle during tetanic contractions, especially ones that lengthen the muscle, which is known to increase intracellular sodium. In disease states such as muscular dystrophy and heart failure, intracellular sodium is generally much higher, poising the exchanger for reversal.

Because the exchanger is a balancer of gradients, a mathematical model should accurately predict its activity based on the energy of the sodium gradient, the energy of the calcium gradient, and the membrane potential. Unfortunately, the result of this basic mathematical approach underestimates both the forward mode and reverse mode activity of the exchanger when intracellular calcium is elevated. This is because of the previously mentioned allosteric activation of the exchanger by calcium. When this allosteric calcium term is included, the activity of the exchanger is much more faithfully modeled (190).

Another important area of NCX1 investigation is the direction of transport that predominates during the cardiac action potential. It was found that NCX1 extrudes calcium for nearly the entire duration of the action potential in wild type cardiomyocytes (191). The duration of forward mode was longer than predicted, as NCX1 is more likely to reverse with depolarization during the action potential. However, subsarcolemmal calcium was found to be much greater than previously realized and this caused the exchanger to act predominantly in forward mode (191). In heart failure, NCX1 reverses for a greater percentage of the action potential due to increased intracellular sodium concentrations (191). The concept of spatially restricted increases in ions may also extend to sodium through the concept of the “fuzzy space” (192). This concept suggests that NCX1 may be regulated by high sodium concentrations that occur near the plasma membrane when sodium channels are open. Understanding the complex
interactions within this diffusion limited space has created special challenges with regard to measurement and is still very much a work in progress.

When NCX1 was discovered, it was very rapidly appreciated that this protein’s actions could explain the very early finding that digoxin, which inhibited sodium pumps, could also increase contractility. NCX1 was the link that could transform elevations in intracellular sodium into increased intracellular calcium concentration; however, when resting intracellular calcium was measured it was only slightly elevated, if at all, by digoxin treatment (193, 194). The key to NCX1’s transformation of sodium pump inhibition into contractility turns out to be the amplifying effect of the sarcoplasmic reticulum (SR). One property of the SR is that the greater the calcium load of the SR, the greater the percentage of calcium released from the SR (195). This property allows relatively small changes in resting calcium due to NCX1 to be translated into larger effects on contractility. While a great deal of progress had been made into the understanding NCX1 biology, genetic studies were required to better understand the role of NCX1 in physiology.

**Role of NCX1 in cardiac physiology**

While early studies of sodium calcium exchange in the heart were very informative, data interpretation was limited by lack of a specific exchange inhibitor or activator. Transgenic and knockout technology provided the tools necessary to study the effects of NCX1 on cardiac physiology. The study of mice with altered expression of NCX1 began with the NCX1 transgenic mouse. The transgenic construct consisted of the canine cardiac NCX1 downstream of the alpha myosin heavy chain promoter (196). These mice exhibited an approximately 3 fold increase in NCX1 activity. They also displayed an accelerated rate of calcium extrusion through the plasma membrane. Surprisingly, despite the large increase of calcium efflux through NCX1,
the SR calcium content and the calcium transient were unaltered. This study was followed by another in the same mice by Yao et. al. (197). Yao found that electrically evoked calcium transients showed only a trend toward increased amplitude, but a significantly longer time to peak. They also found a faster decay of the caffeine evoked calcium transient, similar to the previous study. There was no difference in the activity of the L-type calcium channel, suggesting that there was no compensation when NCX1 was overexpressed. This study also found a faster T50 for the decay of the action potential, but interestingly also found a longer T90, showing enhanced decay of the early part of the action potential but slowed decay of the latter half of the action potential. The data overall suggested that both forward and reverse mode sodium calcium exchange contribute to the action potential.

Interestingly, the phenotype of the NCX1 transgenic mouse very mild unless the mice are stressed severely (196). For example, male NCX1 transgenic mice developed greater dysfunction acutely following ischemia reperfusion injury while female transgenic mice did not (198). This was due to development of alternans, signifying calcium overload, only in male mice. Later studies showed that when the NCX1 transgenic homozygotes were generated, only male mice or post-partum female mice developed worsening disease when exposed to transaortic constriction surgery (199).

A new NCX1 transgenic model recently became available where NCX1 could be overexpressed in an inducible fashion (200). This system allowed bypassing effects on development where NCX1 function has previously been shown to be critical, to determine the relevance of increased sodium calcium exchange in the adult. They also used a rat NCX1 cDNA, which differs from the canine NCX1 that was used in the prior studies. They found that at physiologic calcium levels, contractility and the amplitude of the calcium transient were
unchanged. However at 5 mM extracellular calcium the calcium transient was increased and at 0.6 mM extracellular calcium the calcium transient was greatly decreased. They found that the calcium store was robustly increased at high calcium levels only. The action potential was prolonged in the inducible transgenic but there was no alteration in the L-type calcium channel current. Thus, induced expression of NCX1 shared most characteristics with the constitutive transgenic, especially when similar conditions were compared. Interestingly, the inducible transgenic mice did not develop greater disease than wild type during transverse aortic constriction, which is similar to the results found for the heterozygous constitutive transgenic model.

While the results from the transgenic mice can point the way toward the physiologic role of a protein, the results in the knockout models are thought to be more reliable due to lack of artifacts induced by dramatic protein overexpression. Constitutive NCX1 is embryonic lethal due to a defect in cardiac development (201). Specifically, recent evidence from cardiac pacemaker specific deletion of NCX1 shows that NCX1 is required for the development of a sustained sinus rhythm in mice, which may explain death of mice with constitutive knockouts (202). In contrast to the constitutive knockout of NCX1, deletion of NCX1 utilizing the α-MHC Cre shows no ill effects at baseline. In fact, cardiomyocyte specific deletion of NCX1 is extremely protective in ischemia reperfusion injury, which along with the data showing that NCX1 transgenic mice do worse in ischemia reperfusion, suggests a prominent role for NCX1 in the induction of cellular necrosis (203). Because sodium calcium exchange has long been known to be the most important calcium efflux pathway in the heart, it was very surprising that the NCX1 knockout animals did not develop pathology. Many studies of the physiology of NCX1 knockout animals were devised to understand how this counterintuitive result could occur.
One of the biggest surprises regarding the NCX1 knockout mice was how little the gross physiology was perturbed. Contractility is mildly depressed, in NCX1 deleted hearts, which exhibit increased end systolic dimension, decreased fractional shortening, and decreased ejection fraction (204). Field stimulated calcium transients were unchanged; however, the decay of the caffeine induced calcium transient was prolonged, providing further evidence that NCX1 is able to increased calcium efflux when calcium levels are high (205). The lack of alteration of cardiac physiology in the absence of the major efflux pathway, suggested that compensatory changes occurred. It was found that the duration of the cardiac action potential was markedly shorter in the absence of NCX1 and that L-type calcium channel current was dramatically blunted in the absence of the exchanger (206). It was later found that the decreased L-type current previously observed was coupled to a great increase in the gain of calcium induced calcium release (207). The cardiac myocyte adapted to the lack of a major calcium efflux pathway by decreasing the duration of the action potential, which in turn decreased the duration of L-type calcium channel opening to allow decreased total calcium influx. The decreased calcium influx was further compensated by increased gain of excitation contraction coupling. These results show that sodium calcium exchange is not absolutely required in the adult heart, and also highlight the adaptability of the mammalian cardiomyocyte.

The overall role of NCX1 in the cardiac myocyte remains controversial. It appears that under normal physiologic conditions cardiomyocytes rely on NCX1 for calcium extrusion. While mice only extrude about 7% of the total amount of calcium that enters the cytoplasm during EC coupling, higher mammals such as rabbits, ferrits, guinea pigs, and dogs appear to extrude 25-30% of the total cellular calcium (208). Thus, the lack of dramatic effects found in
the absence of NCX1 might be due to the species studied. Future development of better inhibitors may allow the study of NCX1 in more relevant organisms.

**NCX1 in Skeletal Muscle**

Sodium calcium exchange was first detected in skeletal muscle in 1982 (209). The exchange current in skeletal muscle is approximately 10 fold less than in cardiac muscle (210, 211). However, because skeletal muscle utilizes much less extracellular calcium overall, subtle changes in sarcolemmal calcium regulation can translate into important alterations in contractility. In amphibian skeletal muscle, decreased extracellular sodium concentration was shown to increase contractility. In mammalian muscle, exposure of muscle fibers to ouabain, which inhibits the sodium potassium ATPase, also increases contractility (212). Sodium calcium exchange activity has also been found in individual skeletal muscle fibers, eliminating the possibility that the activity observed in sarcolemmal preparations was from non-muscle cells (213). NCX1 and NCX3 protein and message were also detected in skeletal muscle from rats. Interestingly, NCX1 expression was highest at birth and decreased with subsequent muscle maturation, while NCX3 appeared to increase throughout the life of the mouse. NCX1 was also shown to be expressed most strongly in slow twitch and fast oxidative fibers, while NCX3 was expressed predominantly in fast glycolytic fibers (214). Interestingly, the fast twitch oxidative fibers are largely eliminated in muscular dystrophy and are replaced by immature fibers, slow twitch fibers, and fast oxidative fibers.

Only one study has attempted to measure sodium calcium exchange in dystrophic tissue. They found that reverse mode sodium calcium exchange was much greater in myotubes from DMD boys than from WT controls (215). In this study, the authors found both NCX1 and NCX3
were expressed at similar levels in myotubes from DMD patients and wild type controls. The effects of NCX1 deletion or overexpression on skeletal muscle function have never been studied; however, whole body NCX3 knockouts have been generated. NCX3 deleted animals develop progressive muscular necrosis and have nerve conduction problems. NCX3 was found to localize to the sarcolemma with particular concentration at the neuromuscular junction (216).

Based on all of the above information it is clear that sodium calcium exchange proteins are dynamically regulated in skeletal muscle. It is also clear that sodium and calcium ions are both dysregulated in dystrophic muscle. However, nothing was known about the expression, activity, or functionality of NCX1 in dystrophic muscle, which prompted us to undertake the following work.
Figure 5 – Ionic conditions favoring forward and reverse mode in dystrophic mice. (A) High intracellular sodium concentrations seen in eccentric contraction favor reverse mode sodium calcium exchange. (B) Elevated intracellular calcium concentrations seen in sustained muscle contractions such as aerobic activity favors forward mode sodium calcium exchange. (C) Sodium and calcium concentrations are both elevated in muscular dystrophy and the sodium calcium exchanger 1 (NCX1) is also overexpressed. Because multiple variables are changed, the effect of exchanger overexpression is difficult to assess. (D) In the NCX1 transgenic, the exchanger is overexpressed in isolation.

Chapter 2
Na$^+$ Dysregulation Coupled with Ca$^{2+}$ Entry through NCX1 Promotes Muscular Dystrophy in Mice

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Abstract

Muscular dystrophy and myofiber necrosis typically arise from a diverse series of mutations in genes that normally stabilize the muscle cell membrane (sarcolemma) to prevent
unregulated \(\text{Ca}^{2+}\) entry. Here we generated skeletal muscle-specific transgenic mice expressing the \(\text{Na}^+\)/\(\text{Ca}^{2+}\) exchanger 1 (NCX1) to model a known increase in its expression during muscular dystrophy. The NCX1 transgene induced a dystrophic-like disease state in all hindlimb musculature, as well as exacerbated the muscle disease phenotypes in \(\delta\)-sarcoglycan (\(Sgcd^{-/-}\)), \(Dysf^{-/-}\), and \(mdx\) mouse models of muscular dystrophy. Antithetically, muscle-specific deletion of the \(Slc8a1\) (NCX1) gene diminished hindlimb pathology in \(Sgcd^{-/-}\) mice. Given measured increases in baseline \(\text{Na}^+\) and \(\text{Ca}^{2+}\) in dystrophic muscle fibers, our data suggest that NCX1 produces \(\text{Ca}^{2+}\) entry in hindlimb musculature (but not in the diaphragm). Indeed, \(Atp1a2^{+/-}\) (encodes \(\text{Na}^+\)/\(\text{K}^+\) ATPase \(\alpha_2\)) mice, which have reduced \(\text{Na}^+\) clearance rates, showed exacerbated disease in the hindlimbs of NCX1 TG mice, similar to treatment with the \(\text{Na}^+\)/\(\text{K}^+\) ATPase inhibitor digoxin. Treatment of \(Sgcd^{-/-}\) mice with ranolazine, a broadly acting \(\text{Na}^+\) channel inhibitor, reduced muscular pathology. Our results suggest that inhibitors of reverse mode NCX1 operation, or broadly acting \(\text{Na}^+\) channel inhibitors, could represent novel therapeutic avenues in treating muscular dystrophy.

**Introduction**

Muscular dystrophy (MD) is due to myofiber degeneration that results in muscle loss that ultimately leads to severe functional impairment and/or death. Muscular dystrophies are generally caused by genetic mutations in genes encoding proteins that are either part of the membrane-stabilizing dystrophin-glycoprotein complex (DGC) or otherwise impact some aspect of sarcolemmal integrity (37). Alterations in sarcolemmal integrity then leads to enhanced \(\text{Ca}^{2+}\) entry through micro-tears or stretch-sensitive \(\text{Ca}^{2+}\) channels, or other cation channels (217). Downstream consequences of increased \(\text{Ca}^{2+}\) entry include altered signaling, calpain activation leading to unregulated intracellular protein degradation, and induction of necrosis through
opening of the mitochondrial permeability transition pore with mitochondrial rupture (146, 218). However, the hypothesis that Ca$^{2+}$ elevations directly induce myofiber necrosis and lead to MD is controversial (2). While some groups have indeed reported global or even sub-sarcolemmal increases in Ca$^{2+}$ in dystrophic myofibers (118, 120, 122, 219-221), such measurements are often technically difficult and possibly the reason why other studies have not observed a significant increase (98, 116, 222). However, recent studies in transgenic mice have supported the Ca$^{2+}$ hypothesis for disease. For example, overexpression of dominant negative (dn) transient receptor potential canonical 6 (TRPC6) or dnTRPV2 were each sufficient to abrogate the dystrophic phenotype in mice by inhibiting the type of store-operated Ca$^{2+}$ entry that characterizes these channels (70, 132). Indeed, TRPC3 overexpression in skeletal muscle, which dramatically enhanced Ca$^{2+}$ entry, was sufficient to induce MD in mice (70). Finally, overexpression of the sarcoplasmic reticulum Ca$^{2+}$ ATPase 1 (SERCA1) in skeletal muscle, which dramatically increased the rate of Ca$^{2+}$ clearance back into the sarcoplasmic reticulum (SR), abrogated many pathological indexes of MD in mice (92).

In addition to Ca$^{2+}$, Na$^+$ is also presumed to enter the dystrophic muscle fiber through micro-tears and stretch-activated channels. Importantly, cytosolic Na$^+$ levels appear to be elevated in myofibers from the mdx mouse, a model of Duchenne MD (120, 151, 152, 158, 223). Indeed, recent evidence suggests that intracellular Na$^+$ levels are also elevated in Duchenne MD patients (153, 154). Increased intracellular Na$^+$ could potentially impact dystrophic pathology through cellular edema or reversal of Na$^+$-Ca$^{2+}$ exchange, thereby secondarily leading to even greater Ca$^{2+}$ entry or less clearance (151, 154).

As Ca$^{2+}$ levels rise with contraction some of the Ca$^{2+}$ is removed during relaxation by the Na$^+/Ca^{2+}$ exchanger (NCX) whereby the Na$^+$ gradient generated by the Na$^+/K^+$ ATPase (NKA) is
used to promote Ca\textsuperscript{2+} efflux. While the exchanger typically functions in forward mode to clear Ca\textsuperscript{2+} from the intracellular milieu, reverse mode is also possible leading to Ca\textsuperscript{2+} entry in exchange for Na\textsuperscript{+} (208). The direction of exchange is determined by the Na\textsuperscript{+} gradient, Ca\textsuperscript{2+} gradient, and membrane potential. Forward mode (net Ca\textsuperscript{2+} removal) is favored by low intracellular Na\textsuperscript{+}, high intracellular Ca\textsuperscript{2+} and a hyperpolarized membrane potential. Reverse mode is favored by high intracellular Na\textsuperscript{+}, low intracellular Ca\textsuperscript{2+} and a more depolarized membrane potential. The predominant direction or impact of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange in adult muscle pathology is not currently known, nor is the role of Ca\textsuperscript{2+} entry or efflux by this system understood in MD.

NCX1 function has been more carefully studied in cardiomyocytes that either overexpress or are deleted for the gene encoding NCX1 protein. Inducible overexpression of NCX1 was recently shown to alter cardiac physiology by increasing the Ca\textsuperscript{2+} transient amplitude, increasing myocyte contractility, SR Ca\textsuperscript{2+} content, and increasing the duration of the action potential (224). By contrast, deletion of the gene encoding NCX1 protein had no effect on the amplitude of the Ca\textsuperscript{2+} transient despite decreased L-type Ca\textsuperscript{2+} channel current through increased gain of excitation-contraction coupling (207, 225). NCX1 loss did shorten the action potential duration through an indirect potassium channel-dependent mechanism (206, 226), as well as protect from ischemia reperfusion injury to the heart (203).

Previous studies with NCX isoforms in skeletal muscle suggest that NCX1 is expressed at high levels early in rat embryonic development and postnatal maturation, but is gradually down regulated as NCX3 becomes more highly expressed where it then becomes the primary adult isoform (214). Genetic deletion of the gene encoding NCX3 protein caused myofiber and nerve degeneration that was most prominently demonstrated at the neuromuscular junction (216).
Studies of NCX function in human myotubes from Duchenne patients showed enhancement of reverse mode \( \text{Na}^+ \)-\( \text{Ca}^{2+} \) exchange activity (215). This was also observed in myotubes from dystrophic mice; however, adult muscle fibers failed to show significant reverse mode activity (213, 216, 227). Thus, the role of \( \text{Na}^+ \)-\( \text{Ca}^{2+} \) exchange in MD remains uncertain. Here we show that NCX1 exacerbates MD by facilitating \( \text{Ca}^{2+} \) influx in the limb musculature, but is protective in the diaphragm by facilitating efflux of \( \text{Ca}^{2+} \). Our results provide additional critical evidence for the \( \text{Ca}^{2+} \) hypothesis in MD and suggest novel therapeutic strategies for treating this disease by strategies that lower intracellular \( \text{Na}^+ \).

**Materials and Methods**

**Animals**—Skeletal muscle-specific expression was driven by a modified human skeletal \( \alpha \)-actin promoter with a slow troponin enhancer as described previously (228). This transgenic construct is expressed in both fast and slow twitch muscle but not cardiac or smooth muscle. Canine NCX1 cDNA was used for overexpression because it has been extensively characterized and shown to be allosterically activated by \( \text{Ca}^{2+} \) (unlike mouse), similar to the human protein (190, 229). Seventeen transgenic lines were generated and two were chosen for subsequent analysis based on verification of skeletal muscle-specific expression (low and high line). \( Sgcd^{-/-} \) mice on the C57BL6 background were a gift from Elizabeth McNally (University of Chicago). Both male and female \( Sgcd^{-/-} \) mice were used for analysis. \( mdx \) mice were obtained from Jackson Laboratories. Male NCX1 TG mice were bred to female \( mdx \) mice and male offspring of this cross were utilized for analysis. \( Dysf^{-/-} \) mice were obtained from the Jackson Laboratories (Bar Harbor, Maine) as the A/J strain. \( Atp1a2 \) heterozygous mice were described previously, as were the \( Slc8a1 \) loxP-targeted mice (205, 230). Finally, mice containing the Cre recombinase cDNA
within the myosin light chain1 (Mlc1 gene) locus were also used to achieve muscle-specific gene deletion of appropriately targeted loxP containing alleles (231).

**Western blotting**- Muscle were homogenized in modified RIPA buffer (150 mM NaCl, 50 mM Tris pH=7.4, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 5 mM EDTA, 2 mM EGTA, 1 mM DTT, and 1X Roche Complete Protease Inhibitor). Extracts were cleared by centrifugation at 14,000 g for 10 min. The extracts were then heated for 30 min in Laemmli loading buffer and 5-30 μg of protein was separated on 10% SDS-polyacrylamide gels. Gels were transferred to PVDF membranes that were blocked with 5% milk for 1 hour. Blots were incubated overnight with rotation at 4 ºC with the following antibodies: anti-NCX1 mouse monoclonal at 1:1000 (Swant, Marly, Switzerland, R3F1), anti-NKA mouse monoclonal at 1:1000 (Developmental Hybridoma Database, Iowa City, IA, a5), anti-NKA α1 mouse monoclonal at 1:1000 (Developmental Hybridoma Database, Iowa City, IA, a6f), anti-NKA α2 rabbit polyclonal at 1:1000 (Millipore, Billerica, MA, AB9094), SERCA1 mouse monoclonal at 1:1000. (Affinity Bioreagents, Rockford, IL, MA3911), and dihydropyridine receptor mouse monoclonal at 1:1000 (Pierce, Rockford, IL, MA3912). The blots were washed and subsequently incubated with the appropriate secondary antibody conjugated to alkaline phosphatase at 1:2500. Blots were exposed using chemiluminescence detection reagent (General Electric Healthcare Biosciences, Piscataway Township, NJ).

**Immunohistochemistry and immunocytochemistry**- Whole muscles were excised and immediately immersed in PBS containing 4% paraformaldehyde for 4 h at 4 ºC with agitation. Muscles were then placed in PBS with 150 mM sucrose buffer overnight for cryopreservation. Muscles were bisected, embedded in Optimal Cutting Temperature (VWR, Arlington Heights, IL) compound, and rapidly frozen in liquid nitrogen cooled isopentane. Seven micrometer
Cryosections were cut on a cryo-microtome. Isolated fibers were plated on laminin coated glass bottom cover slips, fixed for 15 minutes in 4% PFA in PBS, and the processed as below. The sections were washed for 5 minutes in PBS, permeabilized for 15 minutes in 0.1% Triton X-100 in PBS and then blocked for 1 h with 5% goat serum in PBS. The sections were incubated overnight at 4 °C with primary antibody dissolved in PBS supplemented with 5% goat serum. The following antibodies and dilutions were used: anti-NKAα2 at 1:100 (Millipore, Billerica, MA, AB9094) anti-NCX1 mouse monoclonal at 1:100 (Swant, Marly, Switzerland, R3F1). The sections were washed 3 times for 5 min in PBS and then blocked for 30 min with 5% goat serum in PBS. Secondary antibody was applied in PBS with 5% goat serum for 1 h at room temperature. Goat anti-mouse secondary antibody conjugated to TRITC and goat-anti-rabbit secondary conjugated to FITC were used at a concentration of 1:400 (Molecular Probes, Eugene, OR). Slides were washed 3 times with PBS for 5 min each. DAPI stain was added for 10 min in the second wash where indicated. Coverslips were mounted with anti-fade reagent and slides were stored at 4 °C until they were imaged on a Nikon A1 laser scanning confocal microscope.

**Photometry experiments** – The flexor digitorum brevis (FDB) muscle was dissected from the footpad in Ringer’s solution (145 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, and 10 mM HEPES, pH=7.4). The FDB was then transferred to a solution containing 0.1% collagenase A for 1 h incubation at 37 °C. Muscles were then washed in Ringer’s solution and single fibers were generated by trituration in the same solution through progressively smaller Pasteur pipettes. Fibers were then loaded with 5 μM Fura-2-AM (Life Technologies, Carlsbad, CA) in Ringer’s solution for 30 min, transferred to a perfusion chamber and allowed to settle for 5 minutes before beginning the experiment. Fibers were excited at 340 nm and 380 nm while emission was detected at 510 nm through a Nikon Ti-U inverted microscope equipped with a 40X Fluor
Forward mode activity measurements were adapted from previous work (213, 216). Baseline readings were acquired in Ca\(^{2+}\) and Mg\(^{2+}\) free (CMF) Ringer’s solution (151 mM NaCl, 5 mM KCl, 10 mM Hepes, pH=7.4) for 1 min. The solution was then exchanged for CMF Ringer’s solution containing 30 μM cyclopiazonic acid (CPA, Sigma Aldrich, St. Louis, MO), 50 μM N-benzyl-p-toluenesulphonamide (BTS, Tocris, Bristol, United Kingdom), and 200 μM EGTA for 100s. The solution was then exchanged again to contain 750 μM 4-chloro-m-cresol (4-CMC, Sigma Aldrich, St. Louis, MO) to elicit Ca\(^{2+}\) efflux through the ryanodine receptor. Traces were recorded for 15 minutes after treatment with 4-CMC. Stimulated transient experiments were performed in Ringer’s solution containing 5 μM BTS. Stimulated transients were evoked by electrical excitation using platinum electrodes at a frequency of 0.2 Hz.

**Histological analysis** - Muscles were bisected at the midpoint of the belly, embedded in paraffin, and cut into 5-7 μm sections. Sections were stained by Masson’s trichrome or H & E. Approximately 1000 fibers per animal were quantified for the presence of centrally located nuclei. Percent blue area was quantified using the Metamorph software package (Molecular Devices, Central Valley, PA).

**Calpain activity experiments** - Calpain activity experiments were performed using the Calpain Glo Protease Assay (Promega, Madison, WI) according to the manufacturer’s instructions. Tibialis anterior muscles were homogenized in ice-cold buffer containing 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 50 mM Tris pH7.4. Lysates were centrifuged at 14,000 g prior to analysis. Samples were diluted to 0.2 μg/μl of protein in the above buffer. Twenty five microliters of sample were combined with 25 μl of Calpain-Glo buffer with Calpain-Glo reagent.
and 2 mM CaCl₂. Samples were incubated for 5 minutes at 37 °C and luminescence was measured at 37 °C using the Synergy 2 plate reader (BioTek, Winooski, VT).

**RT-PCR - Reverse transcription (RT)-PCR.** RNA was isolated using the RNeasy Fibrous Tissue minikit from Qiagen (Valencia, CA) following the manufacturer’s instructions. The RNA was reverse transcribed using the SuperScript III First Strand synthesis kit from Life Technologies (Carlsbad, CA). The cDNA was diluted 1:100, and 5 µl of solution was used per reaction. The results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and quantified using the 2^ΔΔCT method (232).

**Ca²⁺ selective microelectrodes.** Double-barreled Ca²⁺-selective microelectrodes were prepared as described previously (233). The 1.51 mm OD barrel of the pulled microelectrode was back-filled first with the neutral carrier ETH 129 (21193 Fluka-Sigma-Aldrich, St. Louis, MO) and 24 h later with pCa 7 solution. Each Ca²⁺-selective microelectrode was individually calibrated as described previously (234) and only those with a linear relationship between pCa 3 and pCa 7 (Nernstian response, 30.5 mV and 29.5 mV per pCa unit at 37°C and 23°C respectively) were used experimentally. To better mimic the intracellular ionic conditions all calibration solutions were supplemented with 1 mM Mg²⁺ and 8 mM Na⁺. The 0.75-mm OD barrel was backfilled with 3M KCl, just before its use. Interference from Na⁺, and Mg²⁺ on the calibration of these electrodes was negligible (e.g., Na⁺ 20 mM, and Mg²⁺ 3 mM did not alter the response at pCa7). After making measurements of [Ca²⁺], all electrodes were then recalibrated, and if the two calibration curves did not agree within 3 mV, data from that microelectrode were discarded.

**Na⁺ selective microelectrodes.** Double-barreled Na⁺-selective microelectrodes were made as previously described (235). The 1.51 mm OD barrel of the pulled microelectrode was back-filled first with the Na⁺-sensitive ion cocktail (Na⁺ Ionophore I Cocktail A, Fluka Sigma-Aldrich,
St. Louis, MO) based on the neutral ligand ETH-227 and 24 h later with a solution containing 8 mM NaCl. Microelectrodes were calibrated 24h later in solutions containing different [Na\(^+\)] and 1 mM MgCl\(_2\). The microelectrodes gave virtually Nernstian responses at free [Na\(^+\)] between 100 and 10 mM. However, at concentrations between 10 and 1 mM [Na\(^+\)], the electrodes had a sub-nernstian response (40-45 mV), but their response was stable and of a sufficient amplitude to be able to measure [Na\(^+\)]. After making measurements of resting [Na\(^+\)]\(_i\), all electrodes were then recalibrated, and if the two calibration curves did not agree within 3 mV, data from that microelectrode were discarded.

**[Ca\(^{2+}\)]\(_i\) and [Na\(^+\)]\(_i\) determination in mice in vivo**. NCX1 TG and WT mice were anesthetized (ketamine 100 mg/Kg and xylazine 5 mg/Kg), intubated and ventilated with air using a mouse ventilator (Minivent 845, Hugo Sachs Elektronik, Germany). A rectal temperature probe was placed to measure core temperature and to provide a feedback signal to the heating pad (ATC-1000 Temperature controller, WPI) to keep the mice euthermic. A small incision was made in the skin in the back of the left leg, the gastrocnemius muscle was identified and its aponeurosis was partially removed. The superficial fibers were exposed and perfused with rodent Ringer’s solution (37°C). The determination of [Ca\(^{2+}\)]\(_i\) or [Na\(^+\)]\(_i\) were carried out using ion-selective microelectrodes described above.

**Action Potential Measurement** – Action potentials were recorded from fibers within intact extensor digitorum longus (EDL) bundles. Mice were sacrificed by lethal inhalation of isoflurane, the tibialis anterior muscle was dissected away, and suture was tied to the proximal and distal EDL muscle bundle. The bundle was then carefully excised from the mouse and transferred to oxygenated buffer containing NaCl 119 mM; KCl 4.7 mM; CaCl\(_2\) 2.5 mM; MgSO\(_4\) 1.2 mM; KH\(_2\)PO\(_4\) 1.2 mM; NaHCO\(_3\) 25 mM. The ends of the suture were fixed to the glass bottom dish
using super glue. Fibers within the muscle bundle were impaled with a glass recording pipette containing 3 M KCl and recorded with an Axon Patch 200B amplifier. Field stimulation was provided using a Grass stimulator at 0.1 Hz set to deliver a pulse width of 0.1 ms, and the minimum excitation voltage that was capable of eliciting an action potential. Voltage was stepped from 25 V to 50 V until and action potential was achieved. BTS was used at 60 μM to inhibit contraction. A minimum of 3 action potentials were analyzed for membrane potential, amplitude, full duration half maximum, and amplitude of afterhyperpolarization using Clampvit version 10.2.0.12.

**Digoxin Treatment** – NTG and NCX1 TG littermate mice were randomized to a digoxin treatment group or control group at 6 weeks of age. Digoxin in elixir form was produced by Roxane Labs (Columbus, OH) at a concentration of 50 mg/ml and obtained from the Cincinnati Children’s Hospital Pharmacy. Dosage was empirically determined by testing a range of concentrations and obtaining a plasma digoxin concentration from the Cincinnati Children’s Medical Center Pathology Laboratory (Cincinnati, OH). For this study, digoxin was administered in the water by mixing elixir with water in a 1:3 ratio. The treatment was continued for 8 weeks after randomization. Plasma levels were again obtained at the time of sacrifice and determined to be 2 ng/ml on average.

**Ranolazine treatment** – We delivered the drug ranolazine using a diet containing 2 mg ranolazine and 0.18 mg ketoconazole, a P450 inhibitor to reduce ranolazine breakdown, per gram of food, which is based on a diet from a previous study (236). Because adult mice weighing roughly 25 g in our colony consume roughly 4 g of food per day, mice were dosed with 320 mg/kg/d of ranolazine and 28.8 mg/kg/d of ketoconazole. The control chow contained 0.18 mg of ketoconazole per gram of food to control for inhibition of P450 enzymes. Ketoconazole (Teva, Pharmaceuticals, Petach Tikva, Israel) and Ranolazine (Gilead, Foster City, CA) were
obtained from the pharmacy at the Cincinnati Children’s Hospital Medical Center. Mouse food was formulated by Research Diets (New Brunswick, NJ). Mice were treated with drug starting at 4 weeks of age and treatment was continued for 8 weeks.

Statistics - All results and depicted error bars in the graphs are presented as mean ± SEM. Statistical analysis was performed with unpaired 2-tailed t-test (for 2 groups) and 1-way ANOVA with Bonferroni correction (for groups of 3 or more). P values less than 0.05 were considered significant.

Study approval – Human subjects were not used. All experiments involving animals were approved by the institutional animal care and use committee.

Results

**NCX1 Expression is Increased in Dystrophic Skeletal Muscle**

Ca$^{2+}$ handling in the heart and skeletal muscle changes dramatically at birth and with postnatal maturation as these tissues become more rigorously utilized. Not surprisingly, isoform switches occur in many different contractile and Ca$^{2+}$ handling genes to augment contractile function during developmental maturation in both tissues (237). With injury to heart or skeletal muscle there is often a reversion to a more fetal-like gene program in the expression of contractile and Ca$^{2+}$ handling isoforms (238, 239). NCX1 is highly expressed in developing skeletal muscle (214) where it is maintained during the first few weeks of life in rats, but is thereafter strongly down regulated into early adulthood, which we also observed in the mouse (Fig. 5A). However, NCX1 protein expression is maintained during juvenile development up through 6 weeks of age and even into adulthood in dystrophic skeletal muscle from Sgcd$^{-/-}$ mice, which is a mouse model of MD due to loss of the membrane spanning δ-sarcoglycan protein (Fig. 5A). Immunofluorescence staining of histological sections from skeletal muscle of 3-
month-old mice showed very low levels of membrane localized NCX1 protein in wildtype mice (WT), but noticeably higher levels in Sgcd\textsuperscript{-/-} muscles (Fig. 5B). We also observed upregulation of NCX1 after cardiotoxin injury of adult WT skeletal muscle, where new fibers are generated similar to MD (Fig. 5C). Thus, NCX1 is re-expressed or maintained in dystrophic skeletal muscle, but the significance of this on disease is unknown (although it could contribute to the regenerative process in some capacity).

**Figure 5 – NCX1 expression is increased in muscular dystrophy models and in regeneration** - (A) Western blotting for endogenous NCX1 expression at different ages in quadriceps muscle of WT and Sgcd\textsuperscript{-/-} mice. GAPDH is a loading control. Western blot time course is representative of three animals per time point. (B) Histological immunofluorescent images (200X magnification) of NCX1 expression (red) and DAPI (blue) from quadriceps of Sgcd\textsuperscript{-/-} and WT at 3 months of age. (C) Western blotting for endogenous NCX1 during a time...
course of regeneration at 3, 7 and 14 days after cardiotoxin injection into skeletal muscle. Blots are representative of 3 animals per group.

FIGURE 7 – Analysis of NCX1 transgene expression. (A) Schematic human skeletal α-actin (HSA) promoter upstream of canine NCX1 cDNA used to make transgenic (TG) mice. (B) Western blotting in quadriceps and diaphragm of NCX1 TG and Non-transgenic (NTG) mice at 8 weeks of age for NCX1, SERCA1, and NKA (Pan). Western blots are representative of 4 animals per group. Asterisks show difference between the quadriceps and diaphragm in baseline SERCA1 and NKA expression. (C) Immunofluorescence image (200X magnification) of NCX1 (red) versus membranes stained with wheat germ agglutinin (WGA) from histological sections of quadriceps muscle from NTG and NCX1 TG mice. (D) Immunocytochemical staining (600X magnification) of NCX1 (red) and Na+,K+-ATPase α2 isoform (NKAa2, green) in isolated myofibers from NCX1 TG and NTG mice.
Generation of Mice with Increased Skeletal Muscle Specific Expression of NCX1

To determine the effect of sustained expression of NCX1 in MD we generated skeletal muscle-specific transgenic mice expressing the highly characterized canine NCX1 cDNA (196, 229, 240) under the control of the human skeletal α-actin promoter (Fig. 6A). NCX1 overexpression was confirmed in the diaphragm and quadriceps muscle by Western blotting from adult transgenic mice, and proper plasma membrane localization was shown by immunofluorescence (Fig. 6A, 6C and 6D). In isolated fibers, NCX1 was further shown to localize to the t-tubules and endplate similar to NKAα2 (Fig. 6C). These data demonstrate the successful generation of TG mice that overexpress NCX1 as a means of examining its potential functional effects in relation to its known induction in MD.

We next measured Na\(^+\)-Ca\(^{2+}\) exchange activity in isolated FDB muscle fibers from NCX1 TG and WT littermates in a manner similar to those previously described (Fig. 8A) (213, 216). Ca\(^{2+}\) was measured using the ratiometric Ca\(^{2+}\) sensitive dye, Fura-2, excited at 340 nm and 380 nm wavelengths. For this assay, baseline measurements were first obtained for 30 seconds in Ca\(^{2+}\)- and Mg\(^{2+}\)-free Ringer’s solution (CMF). This solution was then exchanged for CMF containing 30 μM cyclopiazonic acid (CPA) to inhibit SERCA function and 50 μM N-benzyl-p-toluene sulphonamide (BTS) to block myosin activity directly and hence contraction for 100s. After 100s, the solution was again replaced with one containing 30 μM CPA and 750 μM 4-chloro-m-cresol (4-CMC), a ryanodine receptor agonist, to elicit Ca\(^{2+}\) release from the SR while maintaining SERCA inhibition (Fig. 8B). Because SERCA is inhibited, the decay of the Ca\(^{2+}\) transient represents Ca\(^{2+}\) efflux from the fibers, which is significantly faster in fibers from NCX1 transgenic mice or dystrophic fibers from mdx mice that also contain the NCX1 transgene (Fig. 1C). To determine if this decay is Na\(^+\) dependent, we performed an experiment exactly as above.
except that Na\(^+\) was replaced with tetraethylammonium chloride (TEA-Cl). The inhibition of the decay of the Ca\(^{2+}\) transient in the absence of Na\(^+\) shows that it is due to the activity of NCX1 (Figure 7). Thus, the transgenic mice express functional NCX1 that greatly increases Na\(^+\)-Ca\(^{2+}\) exchange within the muscle fiber.

To determine the effect of greater NCX1 activity on excitation contraction-coupling we performed field stimulation experiments in WT, NCX1 TG, mdx, and mdx NCX1 TG mice in Ringer’s solution supplemented with 5 \(\mu\)M BTS. Under these conditions we observed a significant increase in the amplitude of the electrically evoked Ca\(^{2+}\) transient (Fig. 8D) in NCX1 TG mice; however, no change in the decay of the Ca\(^{2+}\) transient was observed (Fig. 8E).

Interestingly, NCX1 overexpression had a similar effect on the transient amplitude in both the

![Graph A](image)

![Graph B](image)

**FIGURE 8** - NCX dependence of transient decay phase. (A) Representative traces of forward mode activity in flexor digitorum brevis (FDB) muscle fibers from WT mice at 2 months of age using the Ca\(^{2+}\) sensitive dye Fura-2 in the presence of tetraethylammonium chloride (TEA-CL) shown as the red trace. The black trace is a standard transient in the presence of Na\(^+\) (vehicle). The transient is induced with 4-CMC (arrow) application to cause total ryanodine receptor sensitive Ca\(^{2+}\) release (arrow) and SERCA activity is blocked with cyclopiazonic acid (CPA) in Ca\(^{2+}\) free buffer. (B) Time for ½ of the Fura-2 signal decay of the 4-CMC-induced Ca\(^{2+}\) transient during the forward mode Na\(^+\)-Ca\(^{2+}\) exchange experiment shown in A. Number of fibers analyzed is shown. TEA-CL shows that the decay phase of the transient is due to NCX activity.
WT and *mdx* background. The increased store size suggests that NCX1 could be functioning to facilitate greater Ca\(^{2+}\) entry in the transgenic mice (see below). The observed alterations in Ca\(^{2+}\) handling due to NCX1 overexpression did not result from compensatory changes in SERCA1 or NKA levels in either the quadriceps or diaphragm of TG mice compared with WT (Fig. 6B). However, as will be discussed below, baseline SERCA1 and NKA levels do differ substantially between quadriceps and diaphragm, suggesting that the diaphragm has fundamentally different Ca\(^{2+}\) handling properties from limb musculature (Fig. 6B, asterisks).

We also observed a significant alteration in the membrane voltage relationship of the action potential during repolarization (Fig. 8F). Specifically, action potentials from NCX1 EDL showed increased amplitude of afterhyperpolarization compared with WT fibers, although this relationship was not further altered when crossed into a dystrophic mouse background (Fig. 8F). While not definitive proof this is yet another indirect line of evidence that increased Ca\(^{2+}\) entry occurs in myofibers from NCX1 TG mice given that its known electrogenic properties would hyperpolarize the membrane when acting in reverse mode. Indeed, a description from the late 1980’s already showed that dystrophic fibers from mice lacking laminin-2 displayed membrane hyperpolarization with presumed reverse mode NCX activity (241).
FIGURE 9 – NCX1 overexpression increases Na\(^+\)-Ca\(^{2+}\) exchange. (A) Representative traces of forward mode activity in WT and NCX1 TG (gray and red traces) and mdx and mdx NCX1 (green and blue traces) flexor digitorum brevis (FDB) muscle fibers at 2 to 4 months of age using the Ca\(^{2+}\) sensitive dye Fura-2. 4-CMC is given to cause total ryanodine receptor sensitive Ca\(^{2+}\) release (arrow) and SERCA activity is blocked with cyclopiazonic acid (CPA) in Ca\(^{2+}\) free buffer. (B) SR Ca\(^{2+}\) store calculated from peak height of 4-CMC Ca\(^{2+}\) transient in WT, NCX1 TG, mdx, and mdx NCX1 TG mice. (C) Time for ½ of the Fura-2 signal decay of the 4-CMC-induced Ca\(^{2+}\) transient during the forward mode Na\(^+\)-Ca\(^{2+}\) exchange experiment. *P<0.0001 vs WT. (D) Peak height of an electrically stimulated (paced) Ca\(^{2+}\) transient in Ringer’s solution with 5 μM BTS using the Ca\(^{2+}\) sensitive dye Fura-2. *P<0.001 vs WT. (E) Time constant (Tau) fit to electrically stimulated transients for WT, NCX1 TG, mdx, and mdx NCX1 TG. (F) Amplitude of the afterhyperpolarization of the action potential recorded in WT, NCX1 TG, mdx, and mdx NCX1 TG EDL muscle. *P<0.05 vs WT.

NCX1 Transgenic Mice Develop a Progressive Muscle Pathology in Their Limbs

We initially generated NCX1 transgenic mice under the hypothesis that more exchanger would be adaptive by removing Ca\(^{2+}\) more quickly in the context of MD. However, the data
presented above suggested that NCX1 overexpression in limb muscle fibers was leading to greater Ca\(^{2+}\) influx. Indeed, characterization of muscle histologic and biochemical parameters suggested that NCX1 overexpression induced aspects of a dystrophic phenotype in the limb muscles, which would be consistent with increased Ca\(^{2+}\) entry. Specifically, at two months of age we observed a pseudo-hypertrophic response in the quadriceps and gastrocnemius muscles of TG mice (Fig. 9A), which remained significantly increased in the gastrocnemius at 6 months of age (Fig. 9B). Pseudo-hypertrophy is often associated with MD given ongoing inflammation and fibrosis in the muscle, and this was confirmed by histological analysis at 2, 6 and 15 months of age in NCX1 TG quadriceps (Fig. 9C). Quantitation of histological pathology at 2 and 6 months of age revealed significantly greater fibrosis and central nucleation of myofibers in TG mice compared with non-transgenic (NTG) (Fig. 9D-H). An increase in calpain activity was also observed in NCX1 TG mice, suggesting that necrotic pathways are activated due to greater Ca\(^{2+}\) levels (Fig. 9I). These results show that NCX1 overexpression is sufficient to induce skeletal muscle pathology that is reminiscent of MD.
FIGURE 10—NCX1 overexpression induces progressive skeletal muscle pathology. (A and B) Muscle weight to tibia length (MW/TL) measured at 2 months and 6 months of age in the gastrocnemius (Gastr.) and quadriceps (Quad). *P<0.05 vs NTG. (C) Representative stained histological sections from NCX1 TG and NTG mice at 2 months (H&E, 200X magnification), 6 months (Masson’s trichrome, 100X magnification), and 15 months of age (H&E, 40X magnification). (D and E) Results of hydroxyproline assay to measure collagen content of NTG and NCX1 TG mice at 2 and 6 months of age in quadriceps. *P<0.05 versus NTG. (F and G) Percentage of fibers with centrally localized nuclei at 2 months and 6 months of age in NCX1 TG and NTG histological sections from the indicated muscles. *P<0.05 vs NTG. (H) Percentage of fibers with centrally localized fibers in 6 month-old FDB muscles from NTG and NCX1 TG skeletal muscle. *P<0.05 vs NTG. (I) Calpain activity measured by luciferase assay at 3 months of age in the indicated muscles from NTG and NCX1 TG mice. *P<0.05 vs NTG.
FIGURE 11 – NCX1 overexpression exacerbates hindlimb pathology in Sgcd-/- mice. (A) MW/TL ratio measured in Sgcd-/- and Sgcd-/- NCX1 TG mice at 6 months of age. TA, tibialis anterior; Dia, diaphragm. *P<0.05 vs Sgcd-/- . (B) Hydroxyproline content in quadriceps of Sgcd-/- and Sgcd-/- NCX1 TG measured at 6 weeks and 6 months of age. *P<0.05 vs Sgcd-/- . (C) Histological measurements of area replaced by adipose tissue in quadriceps muscle of Sgcd-/- and Sgcd-/- NCX1 TG mice at 6 months of age. *P<0.05 versus Sgcd-/- . (D) Quantification of TUNEL positive muscle fibers between WT, NCX1 TG, Sgcd-/-, and Sgcd-/- NCX1 TG mice at 6 weeks of age in the quadriceps muscle. *P<0.05 vs WT, #P<0.05 vs Sgcd-/- . (E) Representative H&E and Masson’s trichrome (Tri) images of quadriceps muscle from Sgcd-/-
and Sgcd−/− NCX1 TG mice at 6 months of age. Magnification used is given in the figure. (F) Survival plot of Sgcd−/− and Sgcd−/− NCX1 TG mice.

**NCX1 Overexpression Exacerbates Mouse Models of Muscular Dystrophy**

We observed that NCX1 protein was re-expressed in dystrophic muscle of Sgcd−/− mice well into adulthood, although it was uncertain if this was protective or deleterious in the disease process. To address this issue we first crossed NCX1 TG mice into the Sgcd−/− background. The results showed a much more prominent MD such that by 6 months of age muscle weights were actually significantly lower compared with Sgcd−/− or NTG mice due to extreme necrosis of the myofibers (Fig. 10A, 9B). NCX1 TG Sgcd−/− mice also showed a progressive fibrotic effect that was most prominent at 6 months compared with 6 weeks, likely because endogenous NCX1 is already expressed during the neonatal period (Fig. 10B). Fatty replacement was also greater in NCX1 TG Sgcd−/− mice at 6 months compared with Sgcd−/− alone, which along with the accumulation of fibrosis was clearly visible in representative histological sections from the quadriceps (Fig. 10D). The NCX1 TG also significantly hastened the demise of Sgcd−/− mice, which appears to be due to extreme loss and failure of the musculature as the mice age (Fig. 10E). Overall, these results show that increased NCX1 in the context of MD is deleterious and leads to worsening disease.

To examine the potential “universality” of NCX1 action in MD we also crossed the NCX1 TG to the Dysf−/− and mdx models of disease. Dysf−/− mice are a model of dysferlinopathy associated with defective membrane repair, likely leading to greater Ca^{2+} influx (23). mdx mice lack the protein dystrophin that biochemically models Duchenne MD leading to an unstable membrane and dysregulation in Ca^{2+} (28, 242). We observed that pathological features of limb musculature in Dysf−/− mice became substantially more pronounced in the presence of the NCX1 transgene at 6 months of age (Fig. 11A). Dysf−/− mice with the NCX1 transgene showed
significantly greater loss of quadriceps and gastrocnemius mass at 6 months of age compared with WT, NCX1 TG, or Dysf<sup>−/−</sup> only mice (Fig. 11B), as well as dramatically elevated serum CK levels suggesting greater ongoing rates of muscle breakdown (Fig. 11C). Indeed, rates of myofiber central nucleation were significantly higher in Dysf<sup>−/−</sup> mice containing the NCX1 transgene (Fig. 11D).

**FIGURE 12** – NCX1 overexpression exacerbates pathology in Dysf<sup>−/−</sup> and mdx mouse models. (A) Representative H&E stained histological sections (100X magnification) from gastrocnemius and quadriceps of Dysf<sup>−/−</sup> and Dysf<sup>−/−</sup> NCX1 TG at 6 months of age. (B) MW/TL ratio of WT, NCX1 TG, Dysf<sup>−/−</sup>, and Dysf<sup>−/−</sup> NCX1 TG muscle at 6 months of age. *P<0.05 vs WT; #P<0.05 vs Dysf<sup>−/−</sup>. (C) Serum creatine kinase measurements at 6 months of age in WT, NCX1 TG, Dysf<sup>−/−</sup>, and Dysf<sup>−/−</sup> NCX1 TG mice. *P<0.05 vs WT; #P<0.05 vs Dysf<sup>−/−</sup>. (D) Percent of muscle fibers with centrally localized nuclei in WT, NCX1 TG, Dysf<sup>−/−</sup>, and Dysf<sup>−/−</sup> NCX1 TG quadriceps and gastrocnemius muscles at 6 months of age. *P<0.05 vs WT; #P<0.05 vs Dysf<sup>−/−</sup>. (E) Percent centrally localized nuclei from histological sections in WT, NCX1 TG, mdx, and mdx NCX1 TG mice at 2 months of age. *P<0.05 vs WT; #P<0.05 vs mdx. (F) Percent fibrosis measured by Masson’s trichrome staining from histological sections at 2 months of age in mdx versus mdx NCX1 TG. *P<0.05 vs mdx.

We also crossed NCX1 transgenic mice into the mdx background, which showed greater myofiber central nucleation and fibrosis in the gastrocnemius and quadriceps compared with mdx.
or NCX1 TG only at 2 months of age (Fig. 11E, 11F). This shows that the effect of NCX1 overexpression is conserved over three distinct dystrophic mouse models, suggesting that the deleterious effect of NCX1 overexpression in the hindlimb is not model dependent but a more common feature of MD pathology.

**Deletion of NCX1 Reduces the Dystrophic Phenotype**

Increased expression of NCX1 is clearly detrimental in multiple models of MD but determining the role of endogenous NCX1 expression as a MD disease determinant is also critical. Here we deleted Slc8a1 (NCX1) specifically in skeletal muscle by crossing Slc8a1- loxP-targeted (fl/fl) mice into the Sgcd−/− genetic background. The knock-in MLC-Cre (231) line was utilized to delete Slc8a1 specifically from skeletal muscle of these mice (heart is spared). The Slc8a1-loxP targeted allele was previously shown to be efficiently deleted in the heart when crossed with a cardiac-specific Cre transgenic line, demonstrating its optimal construction (205), and the MLC-Cre allele is known to be robust and efficient in achieving muscle-specific deletion (40). The decreased expression of Slc8a1 mRNA was demonstrated by RT-PCR at 6 weeks of age and was shown to be decreased by approximately 90% (Fig 12A). At 6 weeks of age we observed decreased histopathology with deletion of Slc8a1 specifically in skeletal muscle, including decreased central nucleation in both the quadriceps and the gastrocnemius (Fig. 12B, 12C). Both muscles also showed a large decrease in fibrotic changes with deletion of Slc8a1 in the Sgcd−/− background at this age (Fig. 12D). However, at 6 months of age the protective effect of Slc8a1 deletion was lost, as these double deleted mice progressively showed more disease with aging (Figure 12E). This is probably due to the fact that endogenous NCX1 is only expressed during the neonatal period and is gradually lost by young adulthood. Even in dystrophic mice, NCX1 expression decreases with age, making its deletion less relevant at later
time points. However, the prominent protection observed in 6 week-old Sgcd<sup>−/−</sup> mice with Slc8a1 deletion suggests that early expression of NCX1 in limb musculature contributes to MD disease.

**FIGURE 13** – Deletion of NCX1 (Slc8a1) attenuates skeletal muscle pathology in Sgcd<sup>−/−</sup> mice. (A) Relative mRNA expression of NCX1 and SERCA 1 in Sgcd<sup>−/−</sup> Slc8a1<sup>fl/fl</sup> (control) and Sgcd<sup>−/−</sup> Slc8a1<sup>fl/fl</sup>MLC-Cre quadriceps muscle at 6 weeks of age. (B) Representative muscle histopathology (200X magnification) in Sgcd<sup>−/−</sup> Slc8a1<sup>fl/fl</sup> and Sgcd<sup>−/−</sup> Slc8a1<sup>fl/fl</sup>MLC-Cre mice at 6 weeks of age. Quad, quadriceps; Gastr, gastrocnemius. (C) Percent of centrally localized nuclei quantified from histological sections of the indicated muscles in Sgcd<sup>−/−</sup> Slc8a1<sup>fl/fl</sup> and Sgcd<sup>−/−</sup> Slc8a1<sup>fl/fl</sup>MLC-Cre mice at 6 weeks of age. *P<0.05 vs Sgcd<sup>−/−</sup> Slc8a1<sup>fl/fl</sup>. (D) Percent fibrotic area measured by Masson’s trichrome staining from histological sections of the indicated muscles at 6 weeks of age. *P<0.05 vs Sgcd<sup>−/−</sup> Slc8a1<sup>fl/fl</sup>. (E) Percent central nuclei in gastrocnemius, quadriceps, and diaphragm muscle of Sgcd<sup>−/−</sup> Slc8a1<sup>fl/fl</sup> and Sgcd<sup>−/−</sup> Slc8a1<sup>fl/fl</sup>MLC-Cre mice at 6 months of age. (F) %Fibrosis measured by Masson’s trichrome staining in gastrocnemius, quadriceps, and diaphragm muscle of Sgcd<sup>−/−</sup> Slc8a1<sup>fl/fl</sup> and Sgcd<sup>−/−</sup> Slc8a1<sup>fl/fl</sup>MLC-Cre mice at 6 months of age.
**Intracellular Elevations in Na⁺ Exacerbates NCX1 Mediated Pathology**

Previous studies have demonstrated a small amount of reverse mode NCX activity in myofibers using a technique in which extracellular Na⁺ is replaced with NMDG (213, 216). These and other observations suggested that NCX1 might actually function more dominantly as a Ca²⁺ influx pathway in immature limb myofibers or limb myofibers from adult dystrophic mice. To more specifically address this possibility we first pursued a genetic approach in an attempt to increase reverse mode Ca²⁺ entry. The Na⁺ gradient is a key determinant of net Ca²⁺ influx or efflux, which is directly regulated by the Na⁺-K⁺ ATPase (NKA) pump. Hence, we reasoned that genetic reduction of the NKAα2 gene-product using targeted mice should slightly elevate Na⁺ leading to even greater Ca²⁺ entry from NCX1. Accordingly, we crossed NKAα2 heterozygous mice (Atp1a2⁺/-) into the NCX1 TG background. The α2 isoform of the NKA is the predominant gene expressed in skeletal muscle (243) and is tightly coupled to the activity of NCX (244). We found that NCX1 TG Atp1a2⁺/- mice had increased central nucleation and increased serum CK levels compared with NCX1 TG mice, as well as more obvious histopathology at 3 months of age (Fig. 13B-1C). Moreover, treatment of mice with the NKA inhibitor digoxin, which is known to elevate Na⁺ levels, also significantly exacerbated dystrophic pathology in hindlimb muscle from NCX1 TG mice (Fig. 13D, 13E). These results further support the hypothesis that the adverse effects of NCX1 expression are through enhanced Ca²⁺ entry.
FIGURE 14 – Decreased sodium pump function exacerbates pathology in NCX1 TG mice (D) Representative H&E-stained histological images (200X magnification) of NTG, *Atp1a2*+/−, NCX1 TG, and NCX1 TG *Atp1a2*+/− mice at 3 months of age from the quadriceps. (E) Percent of fibers with centrally localized nuclei at 3 months of age from the same muscles as shown in D. *P<0.05 versus WT; #P<0.05 versus NCX1 TG. (F) Serum creatine kinase levels measured in NTG, *Atp1a2*+/−, NCX1 TG, and NCX1 TG *Atp1a2*+/− mice at 3 months of age. *P<0.05 versus WT; #P<0.05 versus NCX1 TG. (G) Percent centrally localized nuclei in histological sections from quadriceps muscle of NTG-vehicle treated, NTG-digoxin (dig) treated, NCX1 TG-vehicle treated, and NCX1 TG digoxin treated mice. *P<0.05 vs NTG-vehicle treated; #P<0.05 vs NCX1 TG digoxin treated. (H) Percent fibrosis measured by Masson’s trichrome in quadriceps muscle of NTG-vehicle treated, NTG-digoxin treated, NCX1 TG-vehicle treated, and NCX1 TG digoxin treated mice treated for two months starting at 6 weeks of age. *P<0.05 vs NTG-vehicle treated; #P<0.05 vs NCX1 TG digoxin treated

Transgenic NCX1 Expression Rescues Diaphragm Muscle

The NCX1 transgene increased the dystrophic pathology of all hind limb musculature examined. However, when examining the diaphragm we made a key observation that further supported the mechanism of action for NCX1. First, the diaphragm is more specialized to contract rhythmically and hence, has features in common with the heart compared with the rest of the limb musculature (135, 245-247). In the heart, NCX1 is known to operate predominantly in forward mode to remove Ca²⁺ after each contractile cycle, which we suspected could also be the case in the diaphragm. Indeed, we observed that the diaphragm has higher levels of NKA
protein and lower SERCA1 expression compared with the quadriceps, suggesting that the diaphragm is less dependent on internal Ca\(^{2+}\) cycling for contraction and more specialized for sarcolemmal Ca\(^{2+}\) influx and efflux (Fig. 9B). Consistent with these observations, NCX1 overexpression in the diaphragm never led to pathology, and it actually protected Sgcd\(^{-/-}\) mice from dystrophic changes in this muscle at 6 weeks and 6 months of age (Fig. 14A). Quantitation of central nucleation and fibrosis showed significantly lower levels due to the NCX1 transgene in the Sgcd\(^{-/-}\) background (Fig. 14B, C), and there were no longer fibers with Ca\(^{2+}\) deposits due to Ca\(^{2+}\) overload, as assessed histologically with von Kossa staining (Fig. 14D). TUNEL positivity was also decreased in Sgcd\(^{-/-}\) NCX1TG mice further suggesting a protective effect (Fig. 14E). These results provide evidence that NCX1 functions differently in diaphragm versus hindlimb musculature.

NCX1 overexpression driven by the transgene was observed to be relatively lower in the diaphragm versus the quadriceps by Western blot (Fig. 9B). One potential interpretation of the NCX1 transgenic rescue of the diaphragm is that a lower level of NCX1 overexpression is protective, while a higher level of NCX1 overexpression that was observed in the hindlimb becomes deleterious. To determine if this was indeed the case, we generated and crossed a much lower expressing NCX1 transgenic line with Sgcd\(^{-/-}\) mice. Here, as in the high expressing line utilized throughout, slight NCX1 overexpression still rescued the diaphragm at the 6 week time point, but had either no protective effect whatsoever or continued to exacerbate the hindlimb phenotype (Fig. 14F, 14G). Thus, even a smaller increase in NCX1 overexpression in the peripheral musculature is maladaptive toward MD, and still protective in the diaphragm.
FIGURE 15 – NCX1 overexpression rescues histopathology of the diaphragm in MD. (A) Representative H&E stained histological images (40X magnification) from the diaphragm of Sgcd⁻/⁻ and Sgcd⁻/⁻ NCX1 TG mice at 6 weeks of age. (B) Percent of fibers with centrally localized nuclei quantified from diaphragm in Sgcd⁻/⁻ and Sgcd⁻/⁻ NCX1 TG mice. *P<0.05 versus Sgcd⁻/⁻. (C) Fibrotic area (%) from trichrome-stained histological sections of diaphragm at 6 weeks and 6 months in Sgcd⁻/⁻ and Sgcd⁻/⁻ NCX1 TG mice. (D) Images (40X magnification) from von Kossa-stained histological sections of Sgcd⁻/⁻ and Sgcd⁻/⁻ NCX1 TG diaphragm muscle at 6 weeks of age. (E) TUNEL positive myofibers in the diaphragms of WT, NCX1 TG, Sgcd⁻/⁻, and Sgcd⁻/⁻ NCX1 TG mice at 6 weeks of age. *P<0.05 versus Sgcd⁻/⁻.
FIGURE 16 – NCX1 overexpression rescues mdx induced diaphragm pathology

(A) MW/TL ratio measured at 6 months of age in WT, NCX1 TG, mdx, and mdx NCX1TG mice for the indicted muscles. *P<0.05 versus WT; #P<0.05 versus mdx. (B) Representative H&E-stained histological images (40X magnification) of diaphragm from mdx and mdx NCX1 TG mice at 2 months of age. (C) Percent fibrosis measured from Masson’s trichrome-stained histological sections of diaphragm from mdx and mdx NCX1 TG mice. *P<0.05 versus mdx. (D) Percent of fibers with centrally localized nuclei from diaphragm at 6 months of age in mdx and mdx NCX1 TG mice. *P<0.05 versus mdx. (E) Western blots for the indicated proteins from Quadriceps versus diaphragm in WT mice at 6 weeks of age. DHPR, dihydropyridine receptor (L-type Ca2+ channel). Blots are representative of 3 animals per group.

The diaphragm of the mdx mouse displays very substantial pathology that is highly reminiscent of Duchenne MD (248). To extend the clinical relevance of the partial rescue that was observed in the diaphragm of Sgcd−/− mice with the NCX1 transgene, we examined the diaphragms of mdx and mdx NCX1 TG mice at 6 months of age. Examination of muscle weights, as an indication of inflammation and pseudo-hypertrophy disease, showed that the NCX1 transgene increased disease in gastrocnemius and quadriceps, but it had the opposite effect in the diaphragm (Fig. 15A). Gross histological analysis also showed a dramatic protection in the diaphragm of mdx mice containing the NCX1 transgene (Fig. 15B), and quantitation of this effect revealed significantly less centrally localized nuclei and fibrosis at 6 months of age compared with mdx alone (Fig. 15C, D). Thus, two independent models of MD
Figure 17 - Pharmacological treatment of altered Na⁺ decreases dystrophic pathology.
displayed diminished histopathology of the diaphragm, suggesting that NCX1 was protective in this muscle, possibly due to forward mode Ca\(^{2+}\) removal instead of reverse mode Ca\(^{2+}\) entry as occurs in possibly all other musculature (see below). Indeed, as discussed earlier the diaphragm is more specialized for rhythmic contraction, which is consistent with the observation of increased expression of both NKAα1 and NKAα2 isoforms and decreased SERCA expression compared with quadriceps (Fig. 15E). L-type Ca\(^{2+}\) channel levels were unchanged (dihydropyridine receptor, DHPR).

Here we further investigated the association between Na\(^{+}\) and Ca\(^{2+}\) differences in muscle and its role in MD due to NCX1. Interestingly NCX1 overexpression induced an increase in both resting Na\(^{+}\) and Ca\(^{2+}\) in FDB myofibers compared with NTG (Fig. 16A, B). While the increase in Ca\(^{2+}\) did not reach the level of mdx gastrocnemius, it was nearly double the resting Ca\(^{2+}\) level of NTG mice. Surprisingly, mdx NCX1 TG mice showed slightly decreased, though not significant changes in intracellular Ca\(^{2+}\). This may be because the nearly 3-fold elevation in resting Ca\(^{2+}\) in mdx mice shifts the balance towards greater Ca\(^{2+}\) entry by NCX1. It is important to note that this likely does not describe contractile conditions, as the amplitude of stimulated transients was increased in mdx NCX1 TG fibers over mdx fibers (Fig. 8D). With regard to Na\(^{+}\) homeostasis, the NCX1 transgene elevated this ion to nearly an equivalent level to that measured
in myofibers from \textit{mdx} and \textit{mdx} NCX1 TG mice (Fig. 16B). Here, myofibers from \textit{mdx} NCX1 TG mice were found to have a small but significant increase in Na\textsuperscript{+} levels relative to \textit{mdx}. Elevated Na\textsuperscript{+} levels poise a cell to utilize NCX1 as a Ca\textsuperscript{2+} entry mechanism (reverse mode), especially when the cell is depolarized. These results further suggest that NCX1 overexpression shifts the Na\textsuperscript{+}-Ca\textsuperscript{2+} balance in the myofiber towards Ca\textsuperscript{2+} entry, similar to what likely occurs in dystrophic muscle.

The profile of Na\textsuperscript{+} and Ca\textsuperscript{2+} alterations observed in hindlimb myofibers from NCX1TG and dystrophic mice suggested the application of a pharmacologic agent, ranolazine. This drug is known to reduce the leak current of the primary Na\textsuperscript{+} channel expressed in skeletal muscle, Nav1.4, as well as other Na\textsuperscript{+} channels (249, 250). Ranolazine also antagonizes reverse mode Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange in cardiomyocytes through both direct (251) and indirect means (252, 253). Finally, ranolazine is known to result in lower resting intracellular Na\textsuperscript{+} levels in cardiac myocytes (254, 255). Hence, while the profile of this drug is admittedly widespread and not very specific, the fact that it results in lower intracellular Na\textsuperscript{+} and Ca\textsuperscript{2+} appeared ideal for treating MD since these 2 ions are elevated and since there is an increase in NCX1 activity in diseased skeletal muscle fibers in the peripheral musculature. Four-week old \textit{Sgcd}\textsuperscript{-/-} mice were treated with ranolazine in their food at 320 mg/kg/d for 8 weeks (Fig. 16C). Histological examination of quadriceps showed noticeably less pathology with significantly less fibrosis and central nucleation with ranolazine treatment, as well as fewer infiltrates and less fiber size irregularities (Fig. 16D-F). These results not only suggest a novel therapeutic option to one day consider in treating MD, but they also further experimentally suggest that Na\textsuperscript{+} elevation and reverse mode NCX1 contribute to disease.
Discussion

Initial attempts to directly measure Ca\(^{2+}\) levels in dystrophic myofibers or myotubes led to equivocal results, with some observations supporting a general increase in total cytosolic Ca\(^{2+}\) or subsarcolemmal Ca\(^{2+}\) (118, 122, 157, 219, 221) and others showing no difference (98, 116, 222). These divergent accounts may reflect technical difficulties associated with measuring Ca\(^{2+}\) in isolated fibers or myotubes, not to mention the uncertain physiological significance of such measurements in various *ex vivo* preparations or culture conditions. Our direct assessment of Na\(^{+}\) and Ca\(^{2+}\) levels using microprobes showed a significant elevation of both ions in acutely isolated myofibers from *mdx* mice. As a second approach, we and others have adopted a genetic strategy in mice. For example, we showed that overexpression of TRPC3 in skeletal muscle, which mimics/models a known increase in store-operated Ca\(^{2+}\) entry in dystrophic myofibers, directly led to greater Ca\(^{2+}\)-Na\(^{+}\) influx that induced MD in mice (70). Moreover, blocking this known increase in the activity of TRP channels with either a dnTRPC or a dnTRPV construct abrogated MD in mouse models of disease, suggesting that unregulated or greater activation of the TRP class of cation channels is a primary disease determinant. These channels also permeate Na\(^{+}\), which would enhance Ca\(^{2+}\) entry through NCX1 by favoring reverse-mode activity (120, 151, 152, 158, 223). Finally, we also previously showed that overexpression of SERCA1 in skeletal muscle, which produces higher rates of Ca\(^{2+}\) clearance back into the SR, was protective against MD in the mouse (92).

Elevations in Ca\(^{2+}\) lead to myofiber necrosis and disease by at least 2 mechanisms. The first is activation of the Ca\(^{2+}\)-sensitive protease calpain, and the second is mitochondrial swelling and rupture. For the former, Spencer and Mellgren showed that transgene-mediated expression of the calpain inhibitor calpastatin reduced disease in the *mdx* background (256). More recently, it
was shown that mice lacking cyclophilin D (the \textit{Ppif} gene), which regulates calcium dependent necrosis, had decreased pathology and increased strength in several mouse models of muscular dystrophy.

With respect to treatments for human MD, our results strengthen the overall paradigm that Ca$^{2+}$ removal strategies have therapeutic potential. For example, overexpression of SERCA2 in the heart using an adeno-associated virus (AAV) vector has been through clinical trials and has shown early success in treating heart failure by recharging the SR with Ca$^{2+}$ and removing diastolic Ca$^{2+}$ more quickly (257). Thus, a similar strategy, if applied to skeletal muscle, might represent a “universal” approach to treating MD of many different etiologies by reducing myofiber intracellular Ca$^{2+}$ and reducing ongoing necrosis rates. With respect to NCX1, our results suggest that a pharmacologic inhibitor specific for reverse-mode Ca$^{2+}$ entry would be a bona fide therapeutic strategy. Ranolazine might be an appropriate therapeutic agent to consider, as it is known to reduce intracellular Na$^+$ levels, possibly by reducing the leak activity of Nav1.4, as well as possibly by inhibiting reverse-mode NCX1 activity (249-251, 254, 255). Thus, even though this inhibitor is admittedly multifactorial, 8 weeks of ranolazine treatment in \textit{Sgcd$^{-/-}$} mice resulted in significantly less muscle pathology. In addition to these medicinal applications, our study is the first to suggest functional implications of the way in which Na$^+$ overload can be pathological in dystrophic fibers by leading to enhanced Ca$^{2+}$ entry through NCX1.
Chapter 3

Summary, Discussion, and Future Directions
Summary

Previous studies suggested dysregulation of both sodium and calcium homeostasis in muscular dystrophy, but this work remains controversial and very little was known about how concentrations of these ions were balanced by sodium calcium exchange. Here we were able to determine the levels of calcium and sodium in dystrophic skeletal muscle in vivo. We found increased intracellular sodium and calcium concentrations in dystrophic mice. To understand how the balance between these ions occurred in muscular dystrophy we determined the expression level of the sodium calcium exchanger in dystrophic and wild type mice and found it was increased in muscular dystrophy. The overexpression of NCX1 suggested to us that the protein may play an important role in this disease process. Paradoxically, when we measured sodium calcium exchange we found that forward mode sodium calcium exchange was actually decreased in Mdx and Sgcd-/- mice. The most likely cause of decreased sodium calcium exchange observed was the increased intracellular sodium, which diminishes the intracellular to extracellular gradient.

Because the exchanger appears to be overexpressed yet its function was depressed, the generation of a transgenic mouse allowed gain of sodium calcium exchanger function to be more definitively examined in isolation to determine if increased exchange was sufficient to induce pathology. We also overexpressed the canine exchanger, which more faithfully models the human exchanger. The key difference between mouse and canine sodium calcium exchangers appears to be that the canine exchanger is activated by intracellular calcium. This is particularly important because calcium is elevated in muscular dystrophy. Muscle fibers expressing the transgenic exchanger had greatly increased ability to efflux calcium compared to the wild type
exchanger. The transgenic exchanger also increased the activity of sodium calcium exchange in
*mdx* mice.

We found that increased expression of the exchanger produced a very modest level of
disease that increased with aging of the mice. A possible future experiment would involve
measuring resting sodium levels in old versus young mice to understand if increased sodium in
old mice could be driving the dramatic disease that is present in NCX1 TG mice in old age.

In dystrophic models, NCX1 overexpression led to exacerbation of disease in the
hindlimb musculature. The NCX1 TG was shown to be functional in the dystrophic model and
dramatically increased forward mode activity. In this work we also found that sodium levels are
increased in dystrophic mice, which is important because elevated sodium diminishes the driving
force for calcium efflux. This suggests that overexpression of the canine sodium calcium
exchanger with a higher Vmax could compensate for the decreased driving force for sodium
calcium exchange present in dystrophic models. The NCX1 transgene increased disease in both
the *Sgcd*-/ and *mdx* mouse models. This even led to severe atrophy and even death in the *Sgcd*-
/- mouse. Perhaps more interesting was the dramatic exacerbation of disease in the *Dysf*-/ mouse model with overexpression of NCX1. The synergistic increase in severity yields
information about the mechanism of the pathology in both individual disease processes. This
data provides evidence that dysferlin deficiency makes mice dramatically more sensitive to
calcium influx. Future experiments investigating the role of chronic running on the pathology of
the dysferlinopathy and on activity levels on the progress of the human disease may suggest that
limiting activity could be very protective in this disease. Alternatively, this data shows that the
presence of normal repair machinery greatly inhibits NCX1 mediated pathology. Taken together
these studies show that increased NCX1 activity is detrimental to numerous muscular
dystrophies and inhibition of calcium entry through NCX1 may thus be a valid strategy to slow the progress of the pathology in muscular dystrophy.

We also gained information about the mechanism of NCX1TG induced disease mechanisms by treating NCX1 TG mice with digoxin and by crossing them into the α2 NKA +/- background. In the mouse, both of these treatments would have the effect of decreasing the activity of the α2 subunit of the Na-K-ATPase. Skeletal muscle expresses one of the highest levels of NKA-α2 of any tissue. Because this subunit is known to be closely coupled to NCX1, this would have effect of locally increasing sodium concentrations in the vicinity of NCX1, which would enhance reversal of the exchanger. We found that both methods of decreasing activity of the α2 subunit of the Na-K-ATPase dramatically exacerbated disease in the NCX1 TG. This suggests that the NCX1 TG is poised to cause fulminant disease but that it’s held in check by expression of NKA-α2. Future experiments are necessary to determine if these manipulations of the NKA-α2 increase sodium concentrations throughout the cell. It would also be very interesting to know if the exchanger’s activity and directionality is changed by the inhibition of the NKA-α2.

To understand the role of endogenous NCX1 in muscular dystrophy, it was necessary to conditionally delete the exchanger in skeletal muscle. Because the exchanger is overexpressed, yet it’s forward mode function was found to be depressed, it was difficult to predict what the effect of deletion of the exchanger would be. We found that deletion of the exchanger was able to decrease the pathology in Sgcd/-mice. Based on the results of our studies of the concentration of ions in dystrophic skeletal muscle, this is likely to be due to the increased propensity of the exchanger toward reversal in dystrophic skeletal muscle. We found that at 6 months of age, deletion of the exchanger did not decrease pathology. This mirrors the decreased
expression of NCX1 at 6 months. The presence of early but not late rescue may also suggest that NCX1 is more critical during the necrotic phase of the disease. Because there is both a large degree of regeneration and necrosis at the 6 week time points, it would be interesting to understand if NCX1 deletion helps regenerating fibers to mature or if it prevents fully formed fibers from undergoing necrosis. TUNEL staining could point toward whether cell death was inhibited in NCX1 deleted skeletal muscle. Causal evidence could potentially come from in vitro cell death assays performed on deleted cells.

In attempt to translate the finding that NCX1 deletion was protective into a strategy that could potentially applied to humans, we searched for drugs that could inhibit NCX1. We initially experimented with KB-R7493, a drug developed to inhibit NCX1 reverse mode; however, there is very limited in vivo experience with this drug and we did not find a significant difference in pathology when mice were dosed with this drug at 10mg/kg/d two times daily. We next turned our attention to an FDA approved anti-anginal drug ranolazine. Ranolazine works by inhibiting both sodium channels and NCX1. From our perspective this was ideal because could inhibit the exchanger and decrease sodium influx, which may be leading to exchanger reversal. Because this drug was already FDA approved, we knew it could be much more rapidly clinically impactful and there was more in vivo experience in rodents, which allowed us to successfully formulate an effective dose of drug. We found that treatment of mice with ranolazine decreased pathology in Sgcd-/- mice. Future directions for the study of ranolazine in muscular dystrophy include investigating its effect on calcium and sodium influx in muscular dystrophy. This would include studies of the effect of ranolazine on stimulated transients and resting levels of calcium and sodium. It would also be very interesting to perform muscle force testing on ranolazine treated mice to determine if it functionally rescues the disease.
Additionally, studying the effect of ranolazine on survival in *mdx- Utrophin-/-* mice would also provide important evidence prior to progressing this drug to larger animal models. Eventually studies in the golden retriever dystrophy model would be necessary prior to progressing use of this drug in the clinic.

Overexpression of NCX1 decreased fibrosis and central nucleation in the diaphragm of *Sgcd-/-* and *mdx* mice. The rescue observed was hypothesized to be due to alterations in the major calcium handling pathways of the diaphragm in comparison to the hindlimb. The diaphragm has greater expression of sodium ATPase proteins in comparison to the hindlimb. It also appears to have decreased SERCA expressions. This suggests that the diaphragm may rely more heavily on the plasma membrane in calcium handling than hindlimb musculature. The increase in sodium pump prevalence would increase the probability that the sodium calcium exchanger would act in forward mode. Thus, NCX1 may act more prominently in forward mode in the diaphragm. Importantly, we did not observe an increase in the pathology in the diaphragm of mice treated with ranolazine. Based on this data, a drug that could specifically inhibit reverse mode sodium calcium exchange would be ideal for treatment of muscular dystrophy, but until that time, a multi-target agent like ranolazine provides an excellent practical alternative.

**Discussion**

*Sodium dysregulation in dystrophy*

This was only the second study to measure intracellular sodium levels *in vivo* in dystrophic mice, and we found a modestly increased intracellular sodium level in dystrophic mice. We also found that the moderate level of disease induced by NCX1 overexpression was in itself sufficient to elevate sodium levels. The question of the mechanism of sodium elevation in
muscular dystrophy remains, and will require future efforts to solve. The increased sodium may be due to an increase in sodium influx, diminished capacity for sodium efflux, or a combination of these factors. While the sodium pump expression appears to be increased in dystrophic muscle, this does not necessarily mean there is increased pump activity. This is because mitochondrial dysfunction in muscular dystrophy may limit the availability of ATP to the pump. In situ, pump assays are required to answer the question of pump function in dystrophy more definitively. Alternatively the activity of non-specific or sodium selective ion channels may be increased in muscular dystrophy, thus allowing greater sodium influx. Indeed, one study supports increased sodium leak in dystrophic muscles, though further work will be necessary to determine the relevance of this leak to the disease process.

Sodium calcium exchange in skeletal muscle

Prior to this study relatively little was known about sodium calcium exchange in dystrophic muscle. We found an upregulation of the exchanger expression but not exchanger function. We found that the salutary, forward mode sodium calcium exchange rate was greatly decreased in dystrophic muscle. We also showed for the first time that sodium calcium exchange was the dominant mode of calcium efflux from muscle fibers. Thus, the inhibition of calcium efflux may contribute to the calcium overload observed in muscular dystrophy.

This study also was the first to show the impact of increased sodium calcium exchange on skeletal muscle calcium handling. We found that increased sodium calcium exchange led to increased amplitude of the calcium transient, likely by increasing the calcium store size. This is important because the calcium level achieved during every contraction is likely to be higher in NCX1 TG mice, which may further exacerbate calcium overload.
**Effect of NCX1 on action potential**

This was the first study to measure action potentials in single muscle fibers within intact skeletal muscles. The importance of this is that it eliminates artifact that may be introduced by muscle fiber isolation protocols. Because dystrophic muscle fibers are more fragile that WT, there may be a differentially greater damage caused to dystrophic fibers during isolation. By studying the fibers, *in situ*, we avoided the potential for artifact. In contrast to previous reports, we found no difference in the action potential between WT and *mdx* mice. This suggests that the previous changes in action potential observed in *mdx* mice may have been in part introduced through the isolation procedure. It also suggests that the action potential firing mechanism is at least grossly intact in dystrophic muscle.

We also noticed an interesting change in the morphology of the action potentials in NCX1 TG mice. In the NCX1 TG muscles, the afterhyperpolarization was greatly increased. If this is a direct effect of NCX1, this would suggest that NCX1 is acting in reverse or calcium entry mode at the end of the action potential because reverse mode would lead to a hyperpolarizing effect. However, the possibility also exists that this effect is indirect through the upregulation of other channels such as potassium channels, which could also have this effect. Studies of the knockout are warranted to determine if endogenous NCX1 regulates the afterhyperpolarization of the action potential. Additionally, further work using inhibitors such as KBR-7943 could be done to determine whether the effect is the direct result of NCX1 activity or occurs through a compensatory mechanism.
Sodium calcium exchange in disease

This study showed that NCX1 overexpression is deleterious in skeletal muscle. NCX1 TG mice had both increased calpain activity and increased TUNEL positive cells. The increased calpain activity may be due to the increased calcium levels observed in NCX1 TG mice. The increased TUNEL staining reflects greater cell death that occurred in these mice, potentially through increased calpain activity. The end result was greater pathology in NCX1 TG mice. This pathology was dramatically increased by partial inhibition of sodium pumping by genetic and pharmacologic means. This suggests that reverse mode sodium calcium exchange is in itself sufficient to induce a dystrophic phenotype.

Within the dystrophic models, NCX1 TG exacerbated disease. Perhaps the most striking example of this was in the Dysf/- mice where the NCX1 TG transformed a relatively mild disease in the Dysf/- mouse into a fulminant dystrophy. The transformation into a fulminant phenotype may provide mechanistic insight into both of these disease models. The conversion the Dysf/- mice to a fulminant dystrophic phenotype was likely due to increased sodium and calcium levels due to NCX1 overexpression. This suggests that Dysf/- mice are intrinsically sensitive to increased calcium levels. It also suggests that in the absence of normal repair mechanisms in the Dysf/-mice greatly exacerbated the normally mild NCX1 TG phenotype. Future experiments will be necessary to further pursue the role of calcium entry in Dysf/-mice, as these results suggest that they are very sensitive to this ion.

NCX1 TG rescues the diaphragm

An interesting result of this study was that NCX1TG rescued the diaphragm of Sgcd/- and mdx mice. It was initially thought that this may be due to a lower level of transgene
expression in the diaphragm relative to the hindlimb; however, a line with lower expression of NCX1 also rescued the diaphragm and exacerbated the hindlimb pathology further suggesting that this result was due to differences in physiology of the diaphragm and hindlimb and not due to lower expression in the diaphragm. To explain the difference in phenotype of the diaphragm and hindlimb we first profiled the expression of calcium and sodium regulatory proteins in the diaphragm and the hindlimb. We found that the diaphragm had relatively higher expression of membrane proteins such as the sodium potassium ATPase α1 and α2, but lower expression of the SR proteins such as SERCA1. This suggested that the diaphragm was fundamentally different from the hindlimb and may handle calcium to a greater extent using the plasma membrane. We also found that the calcium transient decay was actually faster in the diaphragm despite lower SERCA levels (data not shown) suggesting that the diaphragm may compensate very effectively for a lack of SERCA with membrane proteins. The increased membrane efflux may be necessary in a tissue that rhythmically contracts, as the heart also displays increased levels of calcium efflux through the plasma membrane. Further study will be necessary to define the differences between the diaphragm and the hindlimb and future therapies must be chosen that do not disrupt the diaphragm in favor of rescuing the limb musculature.

**Targeting sodium calcium exchange therapeutically**

After we had seen the effects of NCX1 overexpression we turned our attention to the deletion of NCX1 in skeletal muscle. We found that deletion of NCX1 ameliorated the phenotype in muscular dystrophy. To translate this finding to a potential therapeutic strategy we first tried the reverse mode inhibitor KB-R7943, which partially rescued the phenotype of dystrophic mice but did not yield the robust results we desired. Previous attempts to inhibit NCX1 *in vivo* have had similar difficulty. We then chose to alter NCX1’s activity indirectly by
manipulating sodium homeostasis using the sodium channel inhibitor ranolazine. Ranolazine was effective in robustly decreasing the pathology in Sgcd−/− mice. This drug has been approved by the FDA and thus could potentially be translated to clinical use. One important finding was that ranolazine did not appear to be detrimental to the diaphragm in Sgcd−/− mice. Future work remains to define what functional benefit is gained after Ranolazine treatment. Eventually, translation of this drug to the dog or porcine models of muscular dystrophy would then be necessary prior to a trial in human subjects. Ideally, an improved reverse mode sodium calcium exchange inhibitor will be developed in the coming years, and that would also be another possible therapeutic molecule in treating muscular dystrophy.

Concluding Remarks

The work presented in this thesis validate the concept that dysregulation of intracellular sodium is a critical mechanism of dystrophic pathology, likely feeding into the dysregulation of intracellular calcium. This work also establishes that increased reverse mode sodium calcium exchange is in itself sufficient to produce a dystrophic phenotype. Perhaps the most important finding was that inhibition of sodium channels with ranolazine is a promising therapeutic target for muscular dystrophy. Taken together this work establishes a nodal point for therapy at the interface between dysregulated sodium and calcium concentrations.

Future Direction Summary

*In situ* studies in dystrophic mice to characterize sodium influx and efflux relative to wild type to determine which system is dysregulated in muscular dystrophy.

*Action potential studies of NCX1 deleted animals to determine what effect NCX1 has on the action potential.
*Experiments focusing on calcium handling in the diaphragm and hindlimb to better understand the fundamental physiologic differences between these muscle groups.

*Expression profiling of the diaphragm and hindlimb to better understand alterations in calcium and sodium handling proteins. Future experiments could utilize overexpression strategies to convert the hindlimb to a more diaphragmatic profile.

*Studying the effect of ranolazine on muscle function.

*Determining the mechanism of action of ranolazine in skeletal muscle by assaying sodium influx and sodium calcium exchange with this inhibitor.

*Characterization of NCX1 splice isoform alterations between dystrophic and wild type mice.

*Determining in what context reverse mode sodium calcium exchange occurs in skeletal muscle.


5. !!! INVALID CITATION !!!


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