PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
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TESIS DOCTORAL:

"RESTORATION OF nNOSμ FUNCTION IN THE *MDX* MOUSE MODEL OF DUCHENNE MUSCULAR DYSTROPHY"

Por

DANIELA VICTORIA REBOLLEDO LÓPEZ

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Tesis presentada a la Pontificia Universidad Católica de Chile como parte de los requisitos para optar al grado de Doctor en Ciencias Biológicas mención Biología Celular y Molecular

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Mención Biología Celular y Molecular

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To Waldo,

my partner in great and beautiful adventures…

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ABBREVIATIONS

Abbreviation	Complete description
ALS	Amyotrophic Lateral Sclerosis
BMD	Becker Muscular Dystrophy
cDNA	complementary Desoxiribonucleic Acid
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DGC	Dystrophin Glycoprotein Complex
DIA	Diaphragm
DMD	Duchenne Muscular Dystrophy
DNA	Desoxiribonucleic Acid
ECC	Eccentric Contraction
HA	Hemaglutinine
HRT	Half Relaxation Time
L0	Optimum Length
LGMD	Limb Girdle Muscular Dystrophy
MyHC	Myosin Heavy Chain
NMJ	Neuromuscular Junction
nNOS	neuronal Nitric Oxide Sinthase
NO	Nitric Oxide
RAS	k-Ras membrane destination sequence
RNA	Ribonucleic Acid
SEM	Standard error of the mean
SpF	Specific Force
TA	Tibialis anterior
TPT	Time to Peak Tension
WGA	Wheat Germ Aglutinin
wt	wild type

RESUMEN

La Óxido Nítrico Sintasa Neuronal (nNOS) es una enzima regulada por Ca^{2+}/calmodulina que sintetiza óxido nítrico (ON) desde L-arginina. Existen al menos 4 variantes de *splicing* para nNOS. nNOSμ es una isoforma que posee un inserto de 34 aminoácidos y que es específicamente expresada en músculo esquelético y cardiaco. En el músculo normal, nNOSμ se localiza principalmente en el lado citosólico de la membrana plasmática, el sarcolema, por unión a α-sintrofina, un miembro del complejo glicoproteico asociado a la distrofina (DGC). La localización sarcolemal de nNOSμ es crítica para oponerse a la vasoconstricción simpática y mantener un flujo sanguíneo apropiado a los músculos activos durante el ejercicio.

La expresión, localización sarcolemal y/o la señalización de nNOSμ se encuentran alteradas en varias enfermedades neuromusculares, incluyendo la distrofia muscular de Duchenne (DMD). DMD es un desorden devastador ligado al cromosoma X que comienza en la niñez con dificultad para caminar y correr; progresa con la pérdida de la capacidad ambulatoria en la adolescencia temprana, seguido por perdida de la función de brazos, tronco, y fallas cardiacas y respiratorias. La muerte es común en la adultez temprana. DMD, y el desorden alélico menos severo, la distrofia muscular de Becker, se producen por mutaciones en el gen de la distrofina. En éstas y otras distrofias musculares, existe una necesidad crítica y urgente por tratamientos seguros y eficaces para disminuir la progresión de la enfermedad. Tales terapias reducirán la carga de la enfermedad, mejorarán la calidad de vida y otorgarán tiempo hasta que los tratamientos que se dirigen al defecto genético primario estén totalmente desarrollados, sean económicamente alcanzables y ampliamente distribuidos.

Se ha reportado que la expresión exógena de nNOS puede reducir la severidad de la enfermedad. La expresión citoplasmática de un transgen de nNOSα (la isoforma de nNOS principalmente expresada en neuronas) redujo el daño muscular y la inflamación en el ratón *mdx*, un modelo para DMD; sin embargo, la función muscular de estos animales no fue evaluada. Además, el aumento de la biodisponibilidad de ON puede tener numerosos efectos positivos, pero el solo enriquecimiento no es suficiente. De hecho, la opción terapéutica de incrementar los niveles de nNOSμ sin recuperar su localización sarcolemal se ha convertido en un tema controversial, debido a evidencia mostrando un posible efecto tóxico de un exceso de nNOSμ en el citosol.

El impacto de recuperar la expresión y localización sarcolemal de nNOSμ, la isoforma específica del músculo esquelético, no ha sido evaluado aún. Además, la función y significancia fisiológica del dominio μ, único del *splicing* alternativo de nNOSμ, necesita aún ser investigado.

Nosotros hipotetizamos que nNOSμ es un importante modulador de la función muscular, y que la pérdida de nNOSμ contribuye a la patogénesis de enfermedades neuromusculares, incluyendo DMD. Postulamos que la expresión de un transgen para nNOSμ mejoraría la función muscular en el ratón *mdx*, modelo de DMD, y que el grado de mejora de la patología distrófica depende de la localización de nNOSμ.

Hemos generado, usando inyección pronuclear, ratones transgénicos que expresan nNOSμ o nNOSμ-RAS, un constructo modificado que permite la localización sarcolemal a pesar de la ausencia de distrofina. Estos transgénicos han sido cruzados en el *background* del ratón *mdx*, donde el efecto en la función muscular fue evaluado en diafragma y en el músculo Tibial anterior.

Experimentos funcionales indican que nNOSμ localizado en el sarcolema, pero no nNOSμ citosólico, disminuye la fatiga muscular y el daño inducido con contracciones excéntricas en el músculo esquelético. Por otro lado, la sobre-expresión de nNOSμ en el citoplasma podría llevar a aumentar la susceptibilidad a la fatiga en el diafragma. Nuestra caracterización sugiere que un aumento en la regeneración, un posible desplazamiento en los tipos de fibras hacia fibras menos fatigables, y la recuperación de algunos miembros del DGC en el sarcolema, están entre los efectos que median la mejora de la función muscular.

Nuestros resultados enfatizan la importancia de la localización sarcolemal de nNOSμ, apoyando su papel en el control de la fatiga. Estos son los primeros transgénicos que expresan la isoforma muscular de nNOSμ y por primera vez nNOSμ es dirigido al sarcolema en una forma independiente del DGC. Futuros experimentos comparando con la isoforma neuronal nNOSα proveerán claves acerca del papel de los 34 aminoácidos correspondientes al dominio μ. Además, el logro de la localización sarcolemal de nNOSμ en forma independiente de distrofina permitirá el estudio de la función de nNOSμ en modelos para otros desórdenes neuromusculares donde la enzima se encuentra deslocalizada.

ABSTRACT

Neuronal Nitric Oxide Synthase (nNOS) is a Ca^{2+}/calmodulin-regulated enzyme that synthesizes nitric oxide (NO) from L-arginine. There are at least four splice variants of nNOS. nNOSμ is an alternatively spliced isoform that has a 34 amino acid insert and is specifically expressed in skeletal and cardiac muscles. In normal muscle, nNOSμ localizes mainly to the cytosolic side of the sarcolemma, by binding the dystrophin-associated glycoprotein complex (DGC) member α-syntrophin. The sarcolemmal localization is critical for nNOSμ to oppose sympathetic vasoconstriction and maintain appropriate blood supply to active muscles during exercise. NO also regulates cardiac, skeletal and smooth muscle contractile function and modulates the immune response in muscle.

nNOSμ expression, sarcolemmal localization and/or signaling are impaired in many neuromuscular diseases including Duchenne muscular dystrophy (DMD). DMD is a devastating, X-linked disorder that begins in the toddler years with difficulty with running and progresses to loss of ambulation in the early teens followed by loss of arm, trunk, respiratory and cardiac muscle function. Death is common in early adulthood. DMD, and the milder allelic disorder, Becker muscular dystrophy (BMD), are due to mutations in the gene for dystrophin. In these and other muscular dystrophies, there is an urgent, critical need for efficacious and safe treatments that slow disease progression. Such therapies will reduce disease burden, improve quality of life and buy time until treatments that address the primary gene defect are fully developed, affordable, and widely available.

Exogenous nNOS can reduce disease severity. Cytoplasmic expression of a rat brain nNOSα transgene (the nNOS isoform mainly expressed in brain) reduced muscle damage and

inflammation in the *mdx* mouse, a model for DMD, but muscle function was not tested. Furthermore, enhancement of NO bioavailability may have numerous positive effects, but enrichment alone is not sufficient. Thus, therapeutic option of increasing nNOSμ levels without recovering its sarcolemmal localization have become controversial due to evidence of possible toxic effect of excessive cytosolic nNOSμ.

The impact of restoring skeletal muscle specific nNOSμ isoform expression and localization on dystrophic pathology or muscle function has not been tested. Also the physiological significance and function of the alternatively spliced muscle-specific μ-domain unique to nNOSμ remains to be investigated.

We hypothesize that nNOSμ is an important modulator of muscle function and that the loss of nNOSμ contribute to pathogenesis in neuromuscular diseases, including DMD. We postulate that the expression of a nNOSμ transgene will improve muscle function in the *mdx* mouse model of DMD and that the degree of improvement of dystrophic pathology depends on the localization of nNOSμ.

Using pronuclear injection we generated transgenic mice expressing either nNOSμ or nNOSmu-RAS, a modified construct that allows sarcolemmal localization of the enzyme despite dystrophin absence. These transgenics have been bred onto the *mdx* background, where the effect on Diaphragm and Tibialis Anterior muscle performance was evaluated.

Functional experiments indicate that nNOSμ localized to the sarcolemma, but not cytosolic nNOSμ, decrease the fatigue and eccentric contraction-induced damage in the skeletal muscle. On the other hand, overexpression of nNOSμ in the cytosol could lead to increase fatigue susceptibility in diaphragm. Our characterization suggest that increased regeneration, a possible fiber type shift to less fatigable fibers and restoring of some DGC

members back to the sarcolemma are some of the effects mediating improved muscle performance.

Our results emphasize the importance of nNOSμ sarcolemmal localization, supporting the role of this enzyme in fatigue control. These are first transgenics expressing the muscular isoform of nNOSμ, and for the first time nNOSμ is directed to the sarcolemma in a DGC-independent manner. Future comparison with the neuronal isoform will provide clues about the role of the 34 aminoacids corresponding to the μ domain. Moreover, sarcolemmal localization of nNOSμ in a DGC-independent form will allow the study of nNOSμ function in models for other neuromuscular disorders where the enzyme is misslocalized.

I. INTRODUCTION

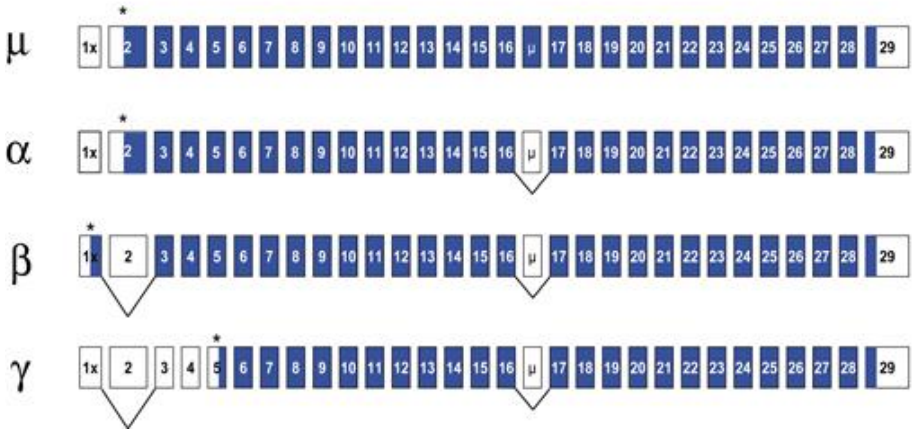
Neuronal nitric oxide synthase (nNOS) is a constitutively expressed Ca^{2+}/calmodulin-regulated protein that catalyzes the synthesis of nitric oxide (NO) from L-arginine. The nNOS protein consists of 2 functional domains: an N-terminal oxygenase domain and a C-terminal reductase domain separated by a calmodulin binding site (Figure 1 and (Zhou and Zhu, 2009)). Alternative splicing of the product of the single nNOS gene leads to the production of different isoforms: nNOSα, nNOSβ, nNOSγ and nNOSμ (Wang et al., 1999).

nNOSα is mainly expressed in brain, in neurons and astrocytes (Zhou and Zhu, 2009). A PDZ domain in the N-terminus of the protein is able to interact with other proteins containing PDZ domains, such as proteins of the postsynaptic density, targeting nNOSα to the plasma membrane (Brenman et al., 1996; Zhou and Zhu, 2009).

nNOSβ and nNOSγ are generated by excluding exon 2 and employing different first exons. Both isoforms lack the PDZ targeting domain and the PIN (Protein Inhibitor of nNOS) binding domain (Figure 1), which is encoded by exon 2; therefore, neither isoform is localized to the plasma membrane. *In vitro* assays have shown that nNOSγ has no significant catalytic activity, while nNOSβ has an activity similar to nNOSα (Brenman et al., 1996; Eliasson et al., 1997).

nNOSμ has a 102-base pair insert between exons 16 and 17, corresponding to a 34 amino acid insert between the calmodulin and FMN (flavin mononucleotide) binding site of the reductase domain. It is specifically expressed in skeletal and cardiac muscles, and its *in vitro* activity as well as its regulation by calcium and calmodulin also seems to be very similar

A.



B.

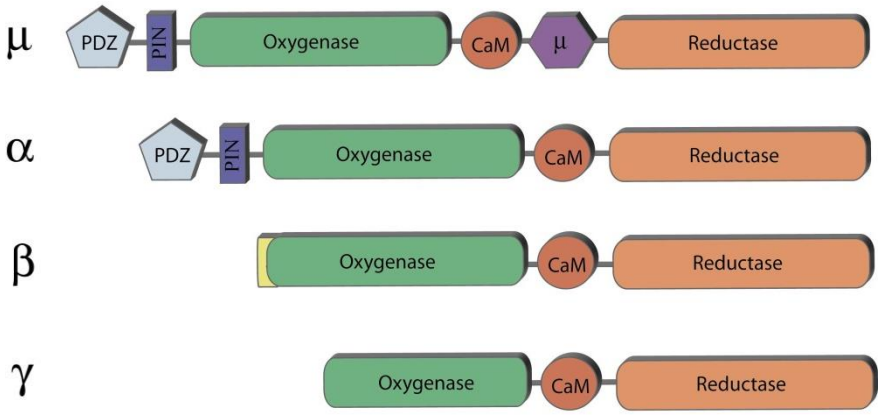


Figure 1: Scheme of nNOS isoforms.

A. Scheme of alternative splicing of nNOS gene. White and blue boxes represent noncoding and coding sequence, respectively. Asterisks represent start codons. **B**. The translated nNOS proteins are represented. PDZ scaffold domain, Protein Inhibitor of nNOS binding domain (PIN), Oxygenase domain, Binding site for Calcium Calmodulin (CaM), μ –insert, and Reductase domain.

to nNOSα (Silvagno et al., 1996). However, these parameters have been measured only *in vitro* with the purified proteins (Silvagno et al., 1996), and the supposed functional equivalence of both isoforms remains a theory. The function of the μ-domain is unknown, but has been postulated to be involved in membrane association and possible posttranslational regulation of nNOSμ (Larsson and Phillips, 1998; Silvagno et al., 1996). For instance, because the rodent μ-domain contains 5 serine residues, it has been proposed as a possible site of regulation by phosphorylation (Silvagno et al., 1996). Interaction with proteins, different or in addition to those interacting with nNOSα, could also be another form of particular regulation for nNOSμ.

nNOSμ and nNOSβ are both coexpressed in skeletal muscle cells and both synthesize NO. NO signals through 2 different pathways. 1) In the cGMP-dependent pathway, NO binds to soluble guanylyl cyclase (sGC), the so-called "NO-receptor", stimulating the conversion of GTP into cGMP. This second messenger binds to downstream effectors that include cGMP-dependent protein kinase (PKG), cGMP-gated channels, and cGMP-regulated phosphodiesterases (Bender and Beavo, 2006; Craven and Zagotta, 2006; Hofmann et al., 2009). 2) In the cGMP-independent pathway, NO can directly react with thiol residues of cysteines (S-nitrosylation) in some proteins, modifying their activity. For instance, the ryanodine (RyR1) Ca^{2+} release channel is activated by S-nitrosylation (Eu et al., 2000).

Although both nNOSμ and nNOSβ splice variants are expressed in muscle cells, they have different subcellular localizations. Recently, nNOSβ was shown to target to the Golgi complex in murine skeletal muscle, where it acts as a critical regulator of muscle structural and functional integrity (Percival et al., 2010). On the other hand, nNOSμ is present in the cytoplasm and is also localized to the inner surface of the sarcolemma, by binding the

dystrophin-associated glycoprotein complex (DGC) member α-syntrophin (Figure 2) (Brenman et al., 1995; Miyagoe-Suzuki and Takeda, 2001).

The roles of nNOSμ in skeletal muscle include regulation of fatigue resistance, maintenance of blood delivery during exercise, control of muscle mass, and modulation of glucose homeostasis (Percival et al., 2008; Ross et al., 2007; Thomas et al., 1998; Thomas et al., 2003; Wehling-Henricks et al., 2009). The sarcolemmal localization is critical for nNOSμ to oppose sympathetic vasoconstriction and maintain appropriate blood supply to active muscles (Thomas et al., 2003). Furthermore, it has been proposed that nNOSμ plays a role in regulating neuromuscular junction (NMJ) structure by increase the expression and clustering of AChRs (Shiao et al., 2004) and may regulate phosphofructokinase (PFK) activity, the rate-limiting enzyme in glycolysis (Wehling-Henricks et al., 2009).

nNOSμ expression, localization and/or signaling are impaired in many neuromuscular diseases of diverse genetic etiology, including Duchenne muscular dystrophy (DMD) (Brenman et al., 1995), Becker muscular dystrophy (BMD) (Chao et al., 1996), Limb-girdle muscular dystrophies (LGMD) 2C, 2D and 2E (Crosbie et al., 2002), Ullrich congenital muscular dystrophy, inflammatory myositis (Kobayashi et al., 2008), muscle atrophy and amyotrophic lateral sclerosis (ALS) (Suzuki et al., 2010; Suzuki et al., 2007). Furthermore, nNOSμ deficiency may contribute to the muscle fatigue that is a common and poorly understood characteristic of many of these diseases, including DMD (Kobayashi et al., 2008; Percival et al., 2010; Schillings et al., 2007). Moreover, myopathic deficits in nNOSμ-deficient skeletal muscle, such as reduced skeletal muscle mass, decreased maximum tetanic force and increased susceptibility to fatigue, suggest that loss of nNOSμ may be contributing to disease severity (Percival et al., 2008). Understanding the molecular mechanisms of muscle

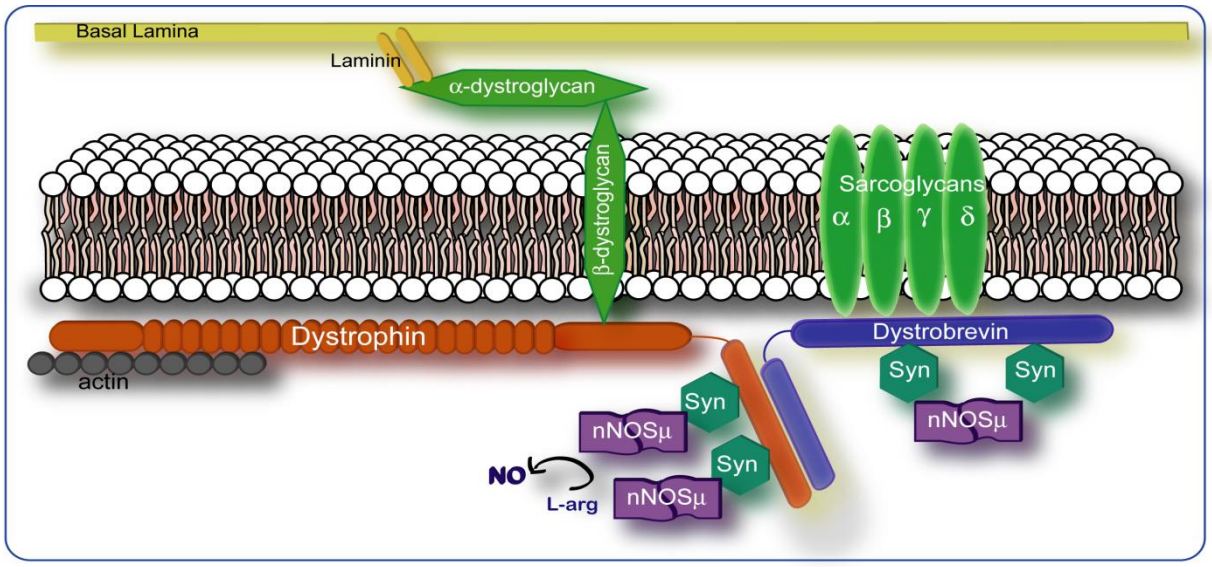


Figure 2. Scheme of the dystrophin-associated glycoprotein complex and nNOSμ.
The DGC in skeletal muscle connects the actin cytoskeleton with the basal lamina. It is composed of dystrophin, dystroglycans (α, β), sarcoglycans (α, β, γ, δ), syntrophins (α, β1) and dystrobrevin (α). A growing number of proteins are reported to be associated with the DGC and have important roles in muscle function and disease, included nNOSμ. Several forms of muscular dystrophy arise from primary mutations in genes encoding components of the DGC. Mutations in dystrophin, all four sarcoglycans and the laminin α2 chain are responsible for DMD/BMD, LGMD type 2C-F and Congenital Muscular Dystrophy, respectively (Durbeej and Campbell, 2002; Ehmsen et al., 2002; Rando, 2001).

fatigue also has relevance beyond DMD and neuromuscular disease to cardiovascular disease and diabetes characterized by exaggerated skeletal muscle fatigue.

DMD is a devastating, X-linked disorder that begins in the toddler years with difficulty with running and progresses to loss of ambulation in the early teens followed by loss of arm, trunk, respiratory and cardiac muscle function. Death is common in early adulthood. DMD, and the milder allelic disorder, BMD, are caused by mutations in the dystrophin gene, which encodes an actin binding protein that links the actin cytoskeleton with the extracellular matrix, forming the dystrophin-associated glycoprotein complex (DGC) (Figure 2) (Durbeej and Campbell, 2002; Ehmsen et al., 2002; Rando, 2001). The dystrophin deficiency leads to loss of other DGC members, including nNOSμ (Ibraghimov-Beskrovnaya et al., 1992; Waite et al., 2009). In both DMD patients and the *mdx* mouse model for DMD nNOSμ expression and activity is greatly decreased and residual nNOSμ cannot localize to the sarcolemma (Brenman et al., 1995; Chang et al., 1996). DMD, as other muscular dystrophies, and its *mdx* mouse model, is also characterized by fibrosis. Fibrosis is an excessive accumulation of extracellular matrix (ECM) components such as collagen and fibronectin (Serrano et al., 2011; Serrano and Munoz-Canoves, 2010; Wynn, 2008) that replaces functional tissue, decreasing muscle force and normal regeneration. Also, increased fibrosis generates a physical barrier that opposes neovascularization, necessary for normal muscle regeneration and function (Gargioli et al., 2008). In DMD and other muscular dystrophies, there is an urgent, critical need for efficacious and safe treatments that slow disease progression. Such therapies will reduce disease burden, improve quality of life and buy time until treatments that address the primary gene defect are fully developed, affordable, and widely available.

Transgenic expression of nNOS in the *mdx* mouse has been reported to improve the histopathology of skeletal muscle, without targeting of the enzyme to the sarcolemma. The cytoplasmic expression of a rat brain nNOSα transgene reduced muscle membrane damage and inflammation in *mdx* muscle (Wehling et al., 2001). They propose an anti-inflammatory function for nNOS where NO protects muscle fibers from damage caused by macrophages (Wehling et al., 2001) plus a role for nNOS to regulate positively the glycolytic metabolism through allosteric modulation of PFK, which could decrease fatigability of *mdx* mice (Wehling-Henricks et al., 2009).

While these results are consistent with a positive impact of nNOS overexpression on the dystrophic histopathology, the approach had several limitations. First, as mentioned before, these studies used nNOSα, the brain isoform rather than the muscle-specific nNOSμ; then, it remains unknown whether endogenous nNOSμ or ectopic nNOSμ expression is also able to perform these protective functions. Second, the enzyme was expressed in skeletal muscle at very high levels (50-250 times normal) (Tidball and Wehling-Henricks, 2004). In addition, the high levels of nNOS in the *mdx* context resided entirely in the cytosol (or at least, not on the sarcolemma), due to the absence of dystrophin and the DGC. Thus, the effects observed might not correspond to the physiological role of the skeletal muscular isoform nNOSμ in its correct localization. Furthermore, this issue becomes important because a toxic gain of function has been suggested for nNOSμ in non-dystrophic muscle when the enzyme is not localized to the sarcolemma: α1-syntrophin-null muscles show displacement of nNOSμ from the sarcolemma and do not regenerate normally (Hosaka et al., 2002), while in tail-suspension, denervation and ALS models, nNOSμ has been shown to be misslocalized and induce muscle atrophy through the activation of Foxo3a and muscle-specific E3 ubiquitin

ligases MuRF-1 and atrogin-1/MAFbx (Suzuki et al., 2010; Suzuki et al., 2007). Finally, no contractile physiological studies were performed to determine if nNOSα expression had a functional effect, positive or negative.

To date, the impact of restoring the expression and localization of skeletal muscle isoform nNOSμ on dystrophic pathology or muscle function has not been tested, and the role of the μ-domain in skeletal muscle nNOSμ remains unknown. We hypothesize that nNOSμ is an important modulator of muscle function and that the loss of nNOSμ contribute to pathogenesis in neuromuscular diseases, including DMD. Therefore, this work aims to define the impact of the restoration of cytoplasmic and sarcolemma-localized nNOSμ on *mdx* skeletal muscle pathology and function.

HYPOTHESIS

nNOSμ improves muscle function in the *mdx* mouse model of DMD depending on its localization.

General Aim

To investigate the potential therapeutic utility of restoring nNOSμ signaling in *mdx* muscle through defining the impact of the restoration of cytoplasmic and sarcolemma-localized nNOSμ on *mdx* skeletal muscle pathology and function.

Specific Aims

1. To a) restore cytoplasmic nNOSμ expression and b) restore sarcolemmal nNOSμ expression in skeletal muscles of *mdx* mouse.
2. To evaluate the localization-dependent- impact of nNOSμ restoration on skeletal muscle pathology and function.

The work displayed in this manuscript includes the generation and primary characterization of new transgenic mice expressing nNOSμ and restoring its sarcolemmal localization. We used molecular biology tools to clone nNOSμ. In order to restore cytoplasmic nNOSμ expression, a nNOSμ transgene was expressed only in the skeletal muscles of *mdx* mice; because of the absence of dystrophin in *mdx* mice, the nNOSμ protein cannot target to the sarcolemma, but only to the cytoplasm. On the order hand, to acchieve sarcolemmal localization of transgenic nNOSμ in the *mdx* context, nNOSμ carrying the k-Ras plasma membrane targeting sequence was expressed in *mdx* skeletal muscles. This construct produces palmitoylated nNOSμ which targets to the sarcolemma, even in the absence of dystrophin. A set of physiological, cell biology and biochemical tools were used to evaluate transgene expression and impact on *mdx* skeletal muscle pathology and function.

The knowledge of the physiological functions of nNOSμ and their dependence on subcellular localization will provide insight into nNOSμ splice variant function in dystrophic muscle. Furthermore, these studies may reveal the usefulness of targeting nNOSμ signaling pathways for therapeutic intervention in muscular dystrophies including DMD and other neuromuscular diseases where nNOSμ signaling is defective.

II. MATERIAL AND METHODS

nNOSμ cloning. Total RNA was extracted from mouse skeletal muscle (mix of hind limb muscles) using the RNeasy Fibrous Tissue Mini Kit (Qiagen). The RNA extracted was used as template to obtain total cDNA using an oligo-dT and SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). nNOSμ was amplified by PCR (AccuPrime *Pfx* DNA Polymerase (Invitrogen) from total cDNA of mouse skeletal muscle using primers nnos5b and nnos3b. A second round of PCR was made with primers designed to insert restriction sites for NotI (5') and PacI (3`) (nos5aNot, nos3aPac). List of primers are listed in Table 1. As cloning vector, we used pBluescript KS- , which has ampicillin resistance and works to perform α-Complementation screening (Sambrook, 2001). pBluescript vector was digested with NotI (NEB) (producing protruding ends) and EcoRV (NEB) (producing blunt ends), while the nNOS PCR product was digested with NotI. The DNA fragments from the restriction reaction were separated by electrophoresis, and the desired bands were gel purified with the Quiaquick Gel Extraction Kit (Qiagen) and ligated with T4 DNA ligase (NEB) (16ºC, ON) into pBluescript KS- vector. The ligation mixture was used to transform electro- or chemically competent *E. coli* DH5α. The transformed bacteria suspension was spread in agar plates supplemented with 100μg/mL ampicillin, 20ug/mL X-gal and 0.1mM IPTG. Individual white colonies were picked with a sterile toothpick or pipette tip to inoculate 14mL falcon tube containing 2mL of LB media, which was shaken at 37ºC ON. The plasmid DNA was purified with a Rapid miniprep protocol.

Rapid miniprep: Suspension of bacteria was centrifuged in a 1.5mL eppendorf tube at maximum speed in a table top centrifuge. Supernatant was discarded by inverting the tube and

Table 1: Primers used for cloning, sequencing and modifing nNOSμ cDNA

Cloning primers	
nnos5b	5’-ccg gag tag ctc agg ttc ctg tg-3’
nnos3b	5’-gtg ggc act cag ggc agc cac-3’
nos5aNot	5’-cct taa tta aca gcc acc cca tat ccc atg-3’
nos3aPac	5’-cct taa tta aca gcc acc cca tat ccc atg-3’
mup1	5’-gtc ttc cac cag gag atg-3’
mup2	5’-aaa ggc aca gaa gtg ggg gta-3’
HA1	5’-cac aga tga ggt ttt cag ctc cta ccc ata tga cgt tcc tga cta cgc atc cct tta att aat taa gg-3’
HA2	5’-gcc tta att aat taa agg gat gcg tag tca gg-3’
HARAS1	5’- gag gtt ttc agc tcc tac cca tat gac gtt c-3’
HARAS2	5’-cgg atc gta tta att aag gcg ccg cgg-3’
Sequencing primers	
Forward 1	5’-gac cag cca tta gca gta gac-3’
Forward 2	5’-ttc tcg acc aat act act c-3’
Forward 3	5’-gtg tcc aac atg ctg ctg g-3’
Forward 4	5’-ctg tgc gag atc ttc aag c -3’
Forward 5	5’-gaa gct cca gag ctg acc-3’
Forward 6	5’-ctc aca cag ctg tcg ctg-3’
Reverse 1	5’-aat ggc cac acc att agc ctg-3’
Reverse 2	5’-tgt cct tga gct ggt agg tg-3’
Reverse 3	5’-agt aat cac gaa cgc caa tc-3’
Reverse 4	5’-ttg cca aag gtg ctg gtg ac-3’
Reverse 5	5’-gaa gat ggt cga tcg gct g -3’
Reverse 6	5’-gta tgg tag gac acg atg gc -3’

pellet was resuspended in the remaining LB media. 200uL of lysis solution (25mM Tris-HCl pH=8; 10mM EDTA pH=8; 0,5N NaOH; 0.2% SDS) was added to each tube and mixed using vortex. Then, lysis reaction was stopped adding 100uL of 5M Sodium Acetate (pH=5) and vortexing. Tubed were centrifuged at maximum speed for 10 minutes. Supernatant was transferred to a new 1.5mL eppendorf tube containing 750µL of 100% ice cold ethanol and centrifuge 10 minutes at maximum speed. Pellet was washed with 500µL of 70% ice cold ethanol and centrifuged for 5 minutes. Pellet was dried and resuspended in TE buffer (100 mM Tris-Cl pH=7.5; mM EDTA pH= 7.5). The plasmid DNA was analyzed by restriction assay using BglII enzyme (NEB). Positive clones showed a characteristic 4 bands pattern. From several procedures, more than 500 colonies were screened, and the positives clones were less than 10. Positives clones were analyzed by PCR to contain µ- or α- nNOS isoform cDNA (mup1 and mup2 primers). This primer pair generates a band of 600pb for nNOSα and 700bp for nNOSµ. Recombinant plasmids carrying the full length nNOSµ cDNA were found, and some of them were sent to sequenciation (Department of Biochemistry, University of Washington). The software Sequencher 4.9 was used to assemble the sequence, and the consensus from this contig was compared with the nNOSµ sequence (Silvagno et al., 1996). The clones showing no differences with the published sequences were chosen.

HA and HA-kRAS tag insertion. To obtain nNOSµ with an HA- tag, two oligos (HA1 and HA2) were designed to delete the original stop codon of nNOSµ, add the HA tag followed by a stop codon and a PacI restriction site. A PCR mix containing oligos HA1, HA2, Forward 6 and nNOSµ in the pBluescript KS- vector was used to obtain the 3' nNOS cDNA carrying the HA tag. To obtain the HA-k-RAS tag, the sequence containing the HA plus the k-Ras (plasma membrane targeting sequence from k-Ras protein amino acid sequence N-KDGKKKKKKSKTKCVIM-C))

tag was amplified from a dystrobrevin-HA-kRas construct using HARAS1 and HARAS2 primers. The HARAS1 primer was designed to add a 15nt sequence that is complementary to 3'end nNOS cDNA. A PCR mix containing oligos HARAS 2, Forward 6 and nNOSμ in the pBluescript KS- vector was used to obtain the 3' nNOS cDNA carrying the HA-RAS tag. In both PCR products, the 3'end of nNOS cDNA plus the respective tags are flanked for SbfI (5') and PacI (3') restriction enzymes, which allowed the exchange of the 3' end portion of the cDNA. The plasmid containing nNOSμ and the PCR products described above were digested with SbfI and PacI. The DNA fragments were purified by electrophoresis and gel extraction as was described above, and the desired fragments were ligated. The ligation mixture was used to transform E. coli DH5α by electroporation. Positives clones carrying the tagged versions of nNOSμ were analyzed by PCR and sequenced to confirm the presence of the corresponding tag. pBluescript KS- plasmids with nNOSμ-HA and nNOSμ-RAS sequences were used to subclone into the NotI/PacI digested pBSX-HSAvpA vector (Crawford et al., 2000) and selected by ampicillin resistance. The nNOSμ transgene was now downstream of the 2.2kb human skeletal actin (HSA) promoter. A VP1 intron sequence from the SV40 virus, containing a transcriptional enhancer, is immediately downstream of the promoter to ensure high transgene expression. Use of the HSA promoter ensures muscle-specific expression of nNOSμ transgenes. Linearized vector (DraI digestion) were injected on zygotes of wild type (C57/Bl6) mouse, and wild type founder mice (C57/Bl6) expressing the nNOSμ transgene were be crossed with wt and *mdx* mice (C57/Bl10) for at least 3 generations.

In situ analysis of skeletal muscle contractile function. Mice were anesthetized with intraperitoneal injections of Avertin (2,2,2, tribromoethanol ; Sigma, St Louis, MO). Mouse hindlimbs were shaved and the distal TA tendon of the tibialis anterior (TA) muscle is surgically isolated via a skin incision on the anterior surface of the lower hindlimb. The mouse was positioned on a 37ºC heated platform in order to restrain the knee joint and the distal

tendon was attached to the lever arm of a servomotor (Model 305B-LR, Aurora Scientific, ON, Canada). The exposed surface of the muscle is kept moist by frequent application of pre-warmed isotonic saline. The TA muscle was stimulated by electrical trigger of the sciatic nerve using two needle electrodes. The muscle was adjusted to an optimum length (Lo) to produce the maximum tetanic force. Then, the time to reach peak tension (TPT) during the contraction phase of the twitch, and the half-relaxation time (HRT), the time between maximum and half maximum force during the relaxation phase of the twitch were recorded. While held at Lo, the TA was stimulated every two minutes at increasing frequencies (10 to 200 Hz) to generate force frequency curves, to obtain the maximal tetanic force (Po). After the completion of testing, both L_0 and TA mass were be recorded and used to normalize to the physiological cross sectional area ([L_0 x density]/mass) and calculate specific twitch (Twitch SpF) or specific tetanic force (SpF). For each mouse, the first hindlimb was used for testing resistance to exercise-induced fatigue, while the second was used to test susceptibility to contraction-induced injury. At the conclusion of contractile function analysis, animals were sacrificed and the tibialis anterior (TA) and diaphragm muscles were rapidly excised, weighed and frozen for analysis.

Resistance to Exercise-Induced Fatigue. To test the capacity of muscle to sustain force output, TA muscles were subjected to a series of repeated contractions to simulate exercise and cause fatigue. Muscles were subject to maximal stimulation (40 V, 200 Hz) at 2 s intervals for 4 minutes. Maximum isometric force production was recorded every 2 s. Recovery from fatigue was assessed by recording maximal tetanic force output every 2 minutes for at least 15 minutes after the completion of the fatigue period.

Resistance to stretch contraction-induced injury. This parameter was assessed by subjecting TA muscles to a series of consecutive lengthening (stretch) of the same strain. Strain is the percentage increase in length beyond the optimal muscle length Lo. Muscles were maximally stimulated (4 V, 200 Hz) for 175ms at fixed length to achieve maximal isometric tension, immediately followed by 175ms of stimulation during the application of a length change of 20% beyond Lo. Strain is applied at the rate of 2 fiber lengths/s. Lengthening contractions were performed at 1 minute interval to minimize the impact of fatigue on force-generating capacity. The isometric SpF generated immediately prior to the initiation of the subsequent lengthening contraction was recorded and normalized.

In vitro analysis of diaphragm muscle function. Diaphragm muscle strips, 2 to 4 mm wide, were dissected in physiological buffer, bubbled with 95% O_2 and 5% CO_2 (pH 7.4). The diaphragm strip was then placed in the experimental chamber, which was continuously perfused with solution. The central tendon of the strip was attached, via a metal clip, to the lever of a dual-mode force transducer/length controller (Aurora Scientific). At the other end of the chamber, the strip was attached to a stainless steel hook, via a small hole in the rib bone. Muscle stimulation was provided by two platinum electrodes, attached to the inside walls of the chamber, which are connected to a stimulator (Aurora Scientific). Supramaximal stimulus voltage is set at 20% greater than the voltage required for maximum twitch force. A length-force curve is then measured by tetanic contractions (120 Hz, 300 ms duration), spaced 1 min apart, over a range of muscle lengths (from short to long). The optimum length (Lo) is the length at which maximum tetanic force is generated. Muscle fiber length (Lf) at Lo is then measured using calipers, for later calculation of specific force (force normalized to muscle physiological cross-sectional area). At this stage, the muscle is subjected to a fatigue protocol.

For fatigue, the muscle is stimulated at 120 Hz, every second for 60 seconds. Recovery of force following fatigue was measured at 1 min intervals up to 15 min.

Western blotting (WB). Equal amounts of protein extracts from transgenic mice were loaded on polyacrilamide gels to perform electrophoresis in presence of SDS (SDS-Page). After electrophoresis, proteins are transferred to a PVDF membrane. The nNOSμ or protein, the HA tag and fibronectin were detected with incubation with primary antibodies at 4°C over night (ON). 3 washes, 10 minutes each, with TBS-T, were performed after primary antibody incubation. Then, PVDF membrane was incubated with HRP-conjugated antibodies per 1h at room temperature (RT), followed by 3 washes with TBS-T. Membrane were developed with ECL (SuperSignal, Thermo Scientific).

Centronucleation and myofiber cross-sectional area (CSA) calculations. Freshly isolated mouse TA and diaphragm muscles were isolated from adult (8-10-week-old) mice, embedded in Tissue Tek OCT compound and flash frozen in liquid nitrogen–cooled isopentane. Cryostat sections (10 μm) from the muscle mid-belly were stained Wheat Germ Aglutinin (WGA-488) and DAPI, and visualized in epifluorescence microscope equipped with a digital camera. Several pictures of the same size and resolution were taken to span the whole TA and approximately ⅓ of the diaphragm. Centronucleation and Fiber Area were measured with Cell Counter and ROI manager plugins of Image J V.1.45s software, respectively.

Immunohistochemistry (IHC). Muscles from 8- to 9-week-old mice were dissected, embedded in Tissue Tek OCT, frozen in liquid nitrogen-cooled isopentane and then stored at -80ºC. Cryostat sections (10μm thick) were dried at room temperature per 5 minutes, and briefly washed in PBS for 1min to eliminate OCT. Samples were fixed in 3% paraformaldehyde for 10 min, and then washed once in PBS per 5 minutes. Slides were

blocked (3%BSA, 2% Fish gel in PBS) for 1h at RT . Commercially available antibodies were used to detect proteins of interest. Primary antibodies were diluted 1:100 in blocking solution to incubate samples 4°C ON. 3 washes, 10 minutes each, with PBS, were performed after primary antibody incubation. Then, slides were incubated Alexa Fluor-conjugated secondary antibodies for 1h at room temperature, followed by 3 washes with TBS-T. Samples were visualized in epifluorescence microscope equipped with a digital camera.

Skeletal muscle fiber typing. To characterize the fiber composition of skeletal muscles, 10-μm-thick frozen sections were immunolabeled with mouse monoclonal antibodies raised against type I (BA-D5), type IIa (SC-71), and type IIb (BF-F3) MyHC proteins (Developmental Studies Hybridoma Bank). Sections were incubated ON at 4°C with primary antibodies followed for 2-hour incubation with Alexa Fluor 350–labeled donkey anti-mouse IgG2B (BA-D5), Alexa Fluor 594–labeled donkey anti-mouse IgG1 (SC-71), and Alexa Fluor 488 donkey anti-mouse IgM (BF-F3) isotype-specific secondary antibodies. The frequency of MyHC type I, type IIa, type IIb, and type IIx/IId fibers per section were counted manually using IMAGE J V1.45s Cell Counter pluggin. Unlabeled fibers were designated type IId/IIx.

Data analysis. In all experiments, transgenic mice were compared to negative littermates in C57Bl10 or *mdx* backgrounds. As different lines were obtained, and results from different lines for the same transgene performed very similar, we used pooled data. Thus, C57Bl10 and *mdx* non transgenic data correspond to negative littermates of all transgenic lines. Data from cytosolic nNOSμ transgenic mice corresponds to the average of 3 different lines. Values are expressed as Mean ± SEM, and statistical differences were evaluated with Prism 5 Software, using One-way ANOVA with Bonferroni multicomparison post-test to evaluate differences between transgenic and non transgenic mice.

III. RESULTS

1. Generation of Transgenic Mice

Transgenic expression of nNOSα in the *mdx* mouse has been reported to improve the histopathology of skeletal muscle (Wehling et al., 2001). However, these studies have several limitations (see Introduction), being the most important the not targeting of the enzyme to the sarcolemma and the use of nNOSα, the brain isoform rather than the muscle-specific nNOSμ. We have taken a different approach to achieve a more normal distribution of nNOSμ in skeletal muscle.

In order to obtain transgenic mice, the cDNA of nNOSμ, and the modified nNOSμ-RAS (carrying the palmitoylation signal from the K-Ras oncogene) were cloned into the pBSX-HSAvpA vector (Crawford et al., 2000), where protein expression is driven by the HSA-promoter, which is active early in embryonic muscle development (Figure 3A). A hemagglutinin tag (HA) was also added. To obtain different lines of the two constructs, several pronuclear injections were performed. After numerous injections and several failed attempts, we successfully detected positive founders for nNOSμ and nNOSμ-RAS transgene by PCR. Founders were crossed with C57Bl10 and *mdx* (same genetic background) mice and the first generation pups (N1) were ear-clipped to genotype them using PCR, analyzing the presence and transmissibility of the transgene. Some of the founders bred successfully, obtaining about 50% of positives pups; however, some of them did not produced pups at all, or if any, all of them were negatives for the transgene, giving evidence that the transgene was not transmitting to the progeny. Positives pups were divided in two groups. First group was kept in order to continue the backcrossing with C57Bl10 or *mdx* until the 3rd generation (N3).

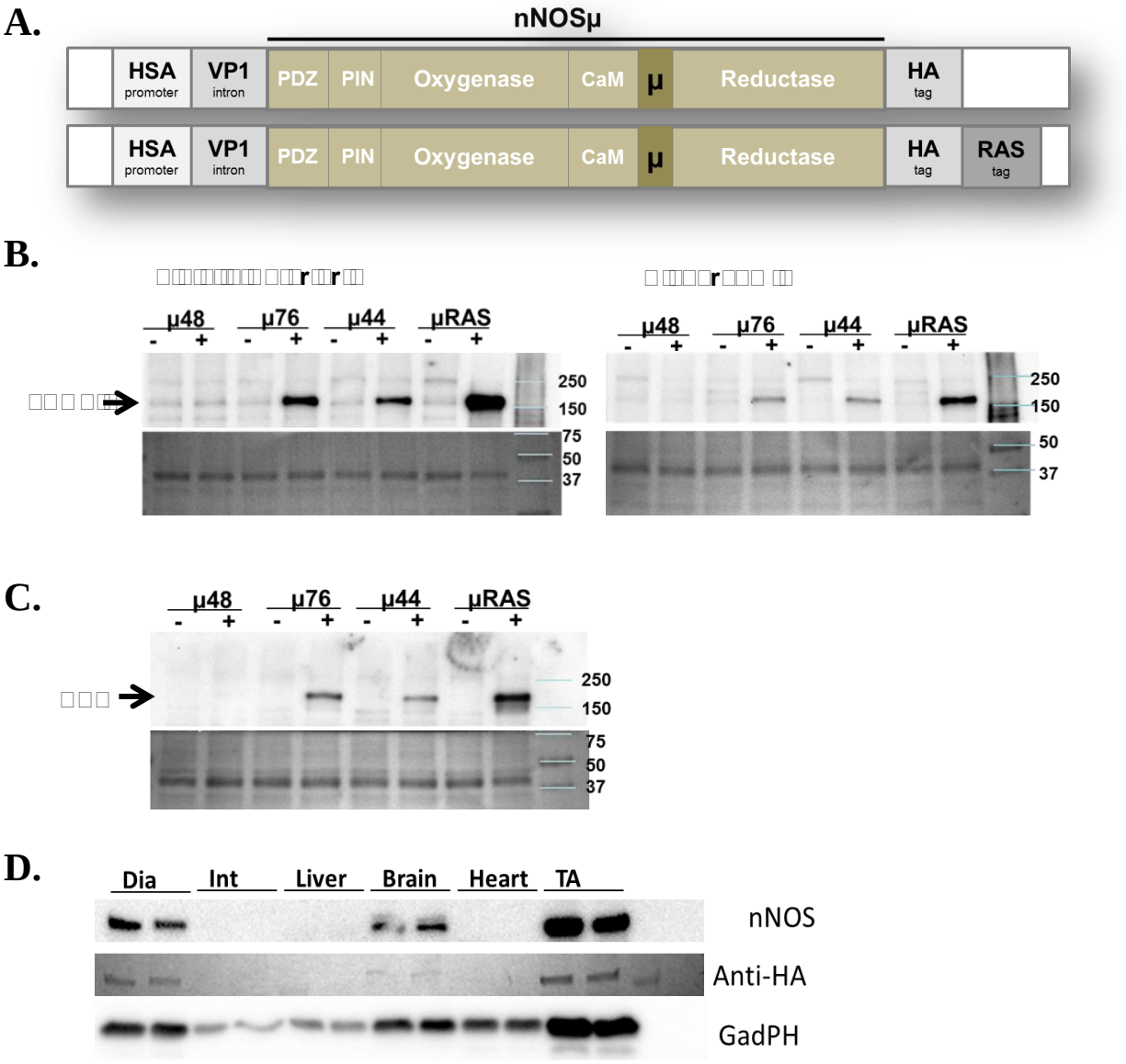


Figure 3. nNOSμ Transgenic Mice

A. Scheme of constructs expressed in transgenic mice. We expressed nNOS containing the μ exon, (the isoform normally expressed in skeletal muscle) with a C-terminal hemagglutinin tag (HA) or the HA tag followed by the palmitylation signal sequence from the RAS oncogene. **B.** Different transgenic lines obtained. Western blot from Tibialis anterior (left) and Diaphragm (right) homogenates using an anti-NOS antibody, showing nNOSμ overexpression. **C.** Western blot from muscle homogenate using an anti-HA antibody, detecting only the transgenic protein. **D.** Western blot from Diaphragm, Intestine, Liver, Brain, Heart, and TA muscle homogenates. Skeletal muscle and Brain show high expression of nNOS (μ and α, respectively). Anti-HA antibody shows only skeletal muscle expression of the transgene.

Second group was sacrificed at 4-6 weeks old to analyze whether the transgene was conducing to protein expression or was silenced. We used western blot to analyze TA and Diaphragm muscle homogenates, and evaluated nNOS expression using an anti-NOS antibody (Figure 3B) and, in order to detect only the transgenic protein, an anti-HA antibody, as both transgenes were designed to carry an HA tag in the N-terminal (Figure 3C). Diverse lines of transgenic mice with different levels of expression were obtained, while other lines were discarded because of null protein expression. Although pBSX-HSAvpA vector and the HSA promoter has been previously used and described to drive only skeletal muscle expression of protein (Crawford et al., 2000), a control western blot using protein homogenates from different tissues was performed to show muscle specific expression of the transgene (Figure 3D).

2. Localization of nNOSμ transgenic protein

In order to evaluate localization of nNOSμ transgenic protein we used 10 μm sections of isopentane-frozen TA muscle for immunofluorescence. Staining against nNOS and the HA-tag shows that mice expressing unmodified nNOSμ can localize the enzyme to the sarcolemma in the *wt* C57Bl10 mice. However, no sarcolemmal staining is seen when the transgene is expressed in the *mdx* background (Figure 4). This is what we expected because of the lack of dystrophin in *mdx* mice. We expected transgenic mice expressing nNOSμ- RAS to be able to localize exogenous nNOSμ to the sarcolemma without the presence of dystrophyn, because of the RAS-tag. In accordance, as is shown in Figure 5, transgenic nNOSμ-RAS protein shows sarcolemmal localization in both *wt* C57Bl10 and *mdx* backgrounds. Same patterns, in lighter staining have been observed in Diaphragm muscle (data not shown).

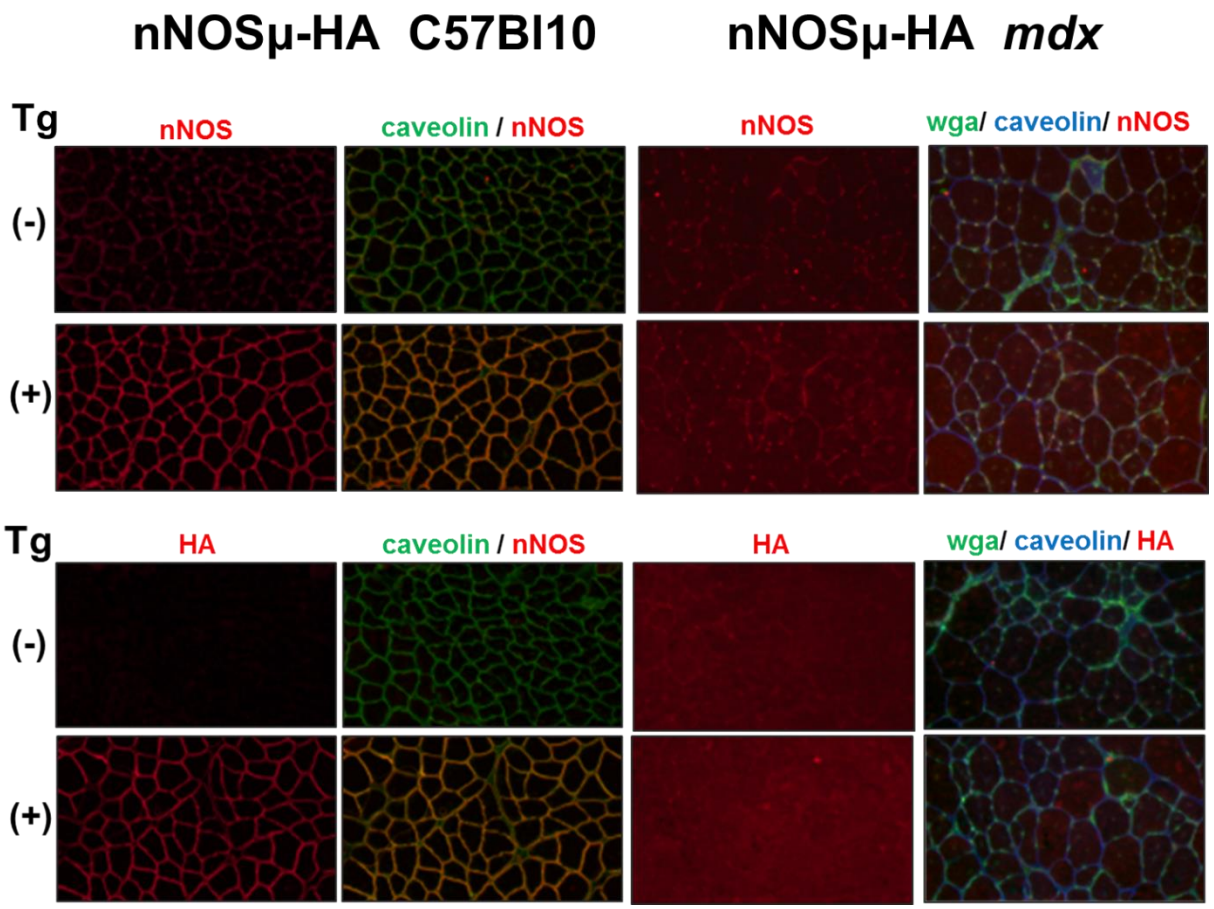


Figure 4. Localization of nNOSμ-HA in C57Bl10 and *mdx* mice

10um sections of isopentane-frozen TA muscle were used for immunofluorescence. Staining against nNOS and the HA-tag were combined with antibody against caveolin and/or wheat germ agglutinin-488 (WGA) to visualize sarcolemma. nNOSμ can localize to the sarcolemma in the *wt* C57Bl10 mice (left) . However, no sarcolemmal staining is seen when the transgene is expressed in the *mdx* background (right).

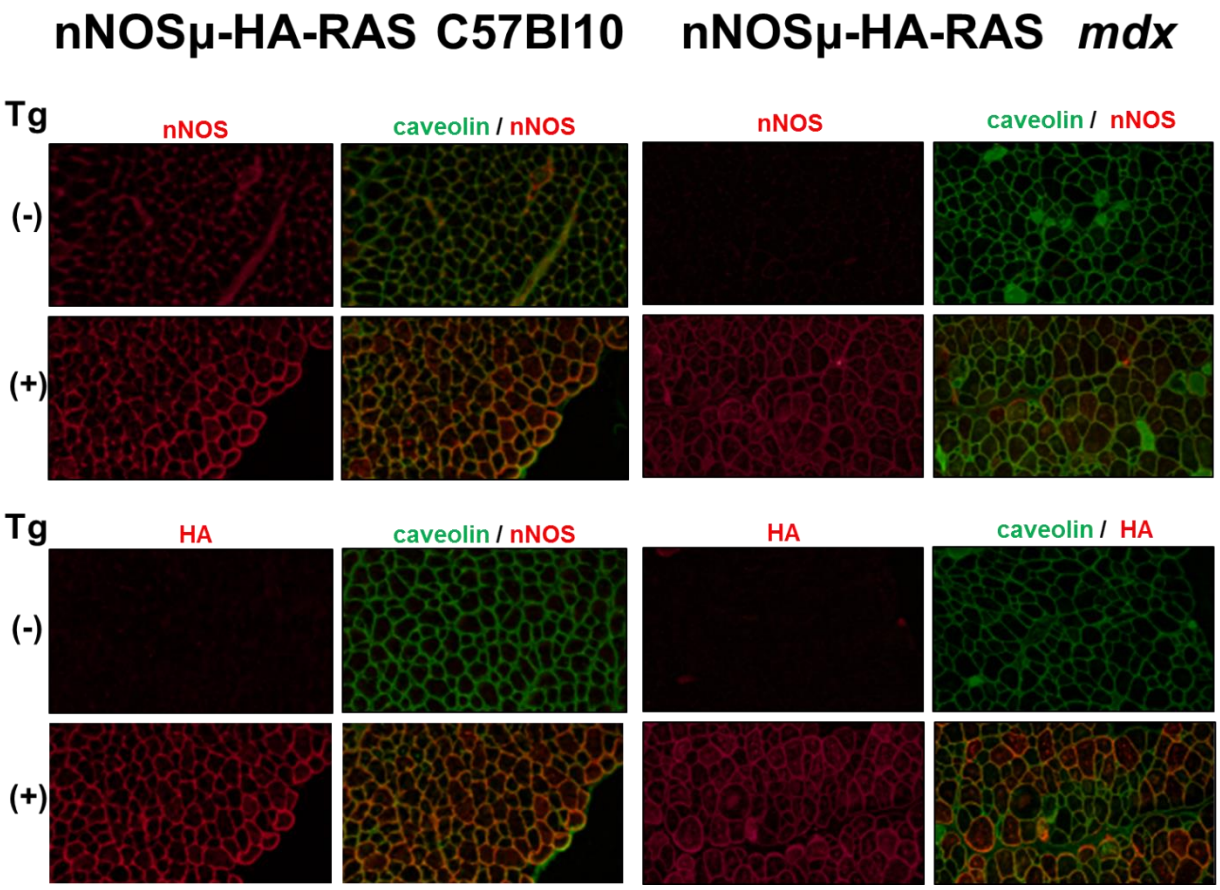


Figure 5. Localization of nNOSμ-HA-RAS in C57Bl10 and *mdx* mice

10um sections of isopentane-frozen TA muscle were used for immunofluorescence. Staining against nNOS and the HA-tag were combined with antibody against caveolin to visualize sarcolemma. Transgenic nNOSμ-RAS protein shows sarcolemmal localization in both *wt* C57Bl10 and *mdx* backgrounds.

A summary panel for nNOSμ localization has been included to compare wt, *mdx* and transgenic lines. As shown in Figure 6, wt C57Bl10 mice show endogenous nNOSμ on the sarcolemma, while *mdx* mouse is deprived of the enzyme. *mdx* mice expressing nNOSμ-RAS have robust amounts of the recombinant enzyme on the sarcolemma. In contrast, the sarcolemma of *mdx* mice expressing unmodified nNOSμ is devoid of the enzyme. Thus, addition of the RAS sequence to nNOSμ alleviates the requirement that both dystrophin and α-syntrophin are needed for sarcolemmal association.

3. Evaluation of Muscle Performance

We evaluated muscle function using two different approaches. First, we used an *ex vivo* evaluation of diaphragm muscle performance as described previously (Whitehead et al., 2008). The *mdx* diaphragm most accurately reflects the severity of the dystrophic phenotype in DMD (Stedman et al., 1991). Since failure of respiratory muscles is a major cause of death in DMD boys, any treatment that improves diaphragm function will likely have therapeutic value and effects on longevity and quality of life. However, we considered this approach could have some limitations when assessing nNOSμ function in skeletal muscle, given the important role of nNOSμ in regulating blood delivery during muscle contraction and the dependence of nNOS function on oxygen concentration (Eu et al., 2003; Thomas et al., 1998; Thomas et al., 2003). Even though controlled buffered solution is used to keep diaphragm strips metabolically active, the conditions, especially oxygen levels, are far from physiological environment. Because of these reasons, we decided to evaluate muscle function with a second

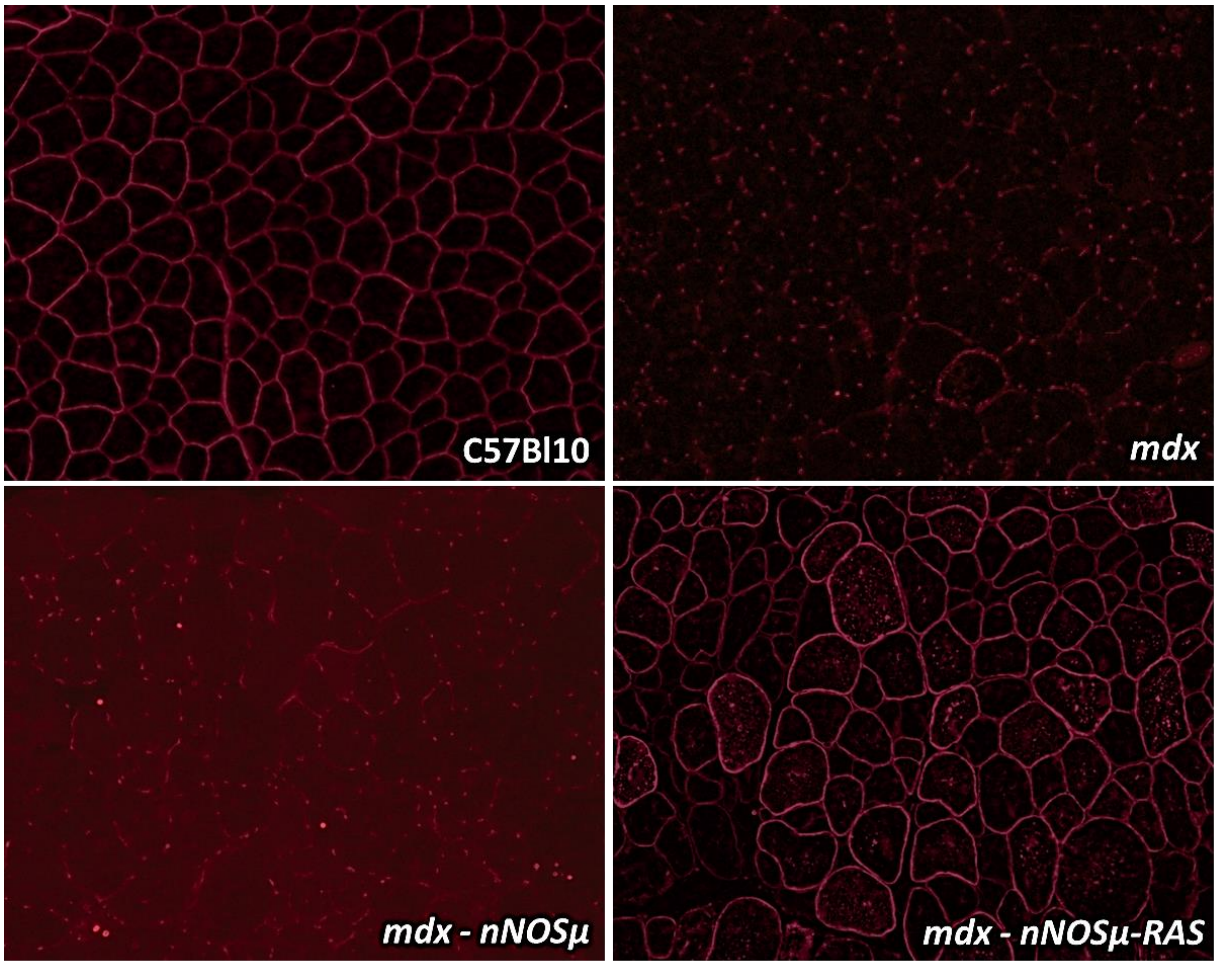


Figure 6. Localization of nNOSμ in transgenic lines.
Muscle cross sections from WT, *mdx*, *mdx* expressing nNOSμ and *mdx* expressing nNOSμ-RAS mice, were immunostained using a nNOS antibody. Note the sarcolemmal nNOS staining only in WT and *mdx* mice expressing nNOSμ-RAS.

approach performing *in situ* physiology analysis of Tibialis anterior (TA) muscle (Percival et al., 2008). The advantage of *in situ* analysis is that it allows for measurement of contractile properties without removing the muscle from its natural environment, maintaining normal vasoregulation and innervation.

We measured different parameters of muscle performance. In both, diaphragm *ex vivo* and TA *in situ* techniques, we measured Specific Force (SpF). SpF is the maximum tetanic force the muscle can develop in its optimal length (L_0), normalized to the physiological cross sectional area ([L_0 x density]/weight). When calculating SpF in penniform muscles (those in which muscle fascicles attach obliquely to the tendon) as the TA, L_0 must be corrected by the pennation angle. Pennation angle is formed by the individual muscle fibers with the line of action of the muscle, and the used value is an average for the entire muscle, which in TA correspond to a value of 0.6 (Hakim et al., 2011). At the same L_0, twitch Specific force (SpF generated after a single stimulation) was measured. From these records, parameters as the time to reach peak tension (TPT) during the contraction phase of the twitch and the half-relaxation time (HRT), the time between maximum and half maximum force during the relaxation phase of the twitch, can also be analyzed as a measurement of the kinetics of the muscle contraction.

To evaluate muscle function during activity, we performed a protocol that pretends to mimic what happens, for example, during exercise. This protocol is used to evaluate muscle fatigue susceptibility, and was performed in both Diaphragm and TA muscles (see Methods) (Percival et al., 2008). Muscles are subjected to consecutive tetanic stimulations every 1 or 2 seconds; muscle fatigues and force decreases after each contraction. Then, tetanic stimulations are performed every 1 minute for at least 10 minutes, to evaluate muscle force recovery.

3.1 Ex vivo evaluation of Diaphragm Performance

We evaluated the diaphragm muscle performance as described previously (Whitehead et al., 2008) using small (2-4 mm wide) strips of tissue, trying to maintain intact fibers that go from the rib bone to the central tendon of the diaphragm. After determination of L_0, the SpF was measured at frequencies of 10, 30, 50, 80, 100 and 120Hz, and SpF vs Frequency was plotted comparing control and transgenic lines. Expressions of nNOSμ or nNOSμ-RAS have not effect on SpF in C57Bl10 or *mdx* mice (Figure 7A). We also measured the twitch specific force, the force evoked for a single stimulus. Again, expression of nNOSμ or nNOSμ-RAS does not affect the twitch SpF in C57Bl10 or mdx diaphragm (Figure 7B).

After determining the specific force, we performed a fatigue protocol. The diaphragm strip was subjected to a tetanic stimulation (120 Hz) every 1 second for 1 minute. This first step decreases muscle force after each contraction. Then, in a second step, a tetanic stimulation was performed every 1 minute for at least 10 minutes, to evaluate force recovery. When expressed in C57Bl10, nNOSμ or nNOSμ-RAS do not change the fatigue or the recovery phase of the protocol (Figure 7C). This result suggests that over expression of nNOSμ has no effect on fatigue susceptibility in non-dystrophic diaphragm. Same experiments were performed on diaphragm of nNOSμ transgenic mice in the *mdx* background. We found that transgenic mice expressing nNOSμ in the cytosol show slightly more diaphragm fatigue susceptibility than the *mdx* negative littermates, which can be seen in both fatigue and recovery steps of the protocol. On the other hand, diaphragm from *mdx* mice expressing nNOSμ-RAS show similar force decay than control *mdx* mice, but they recover faster and reach the 100% of the initial force, a value that is never seen in a regular *mdx* mouse (Figure 7D). This result suggest that sarcolemmal localized nNOSμ allows to reduce fatigability, and

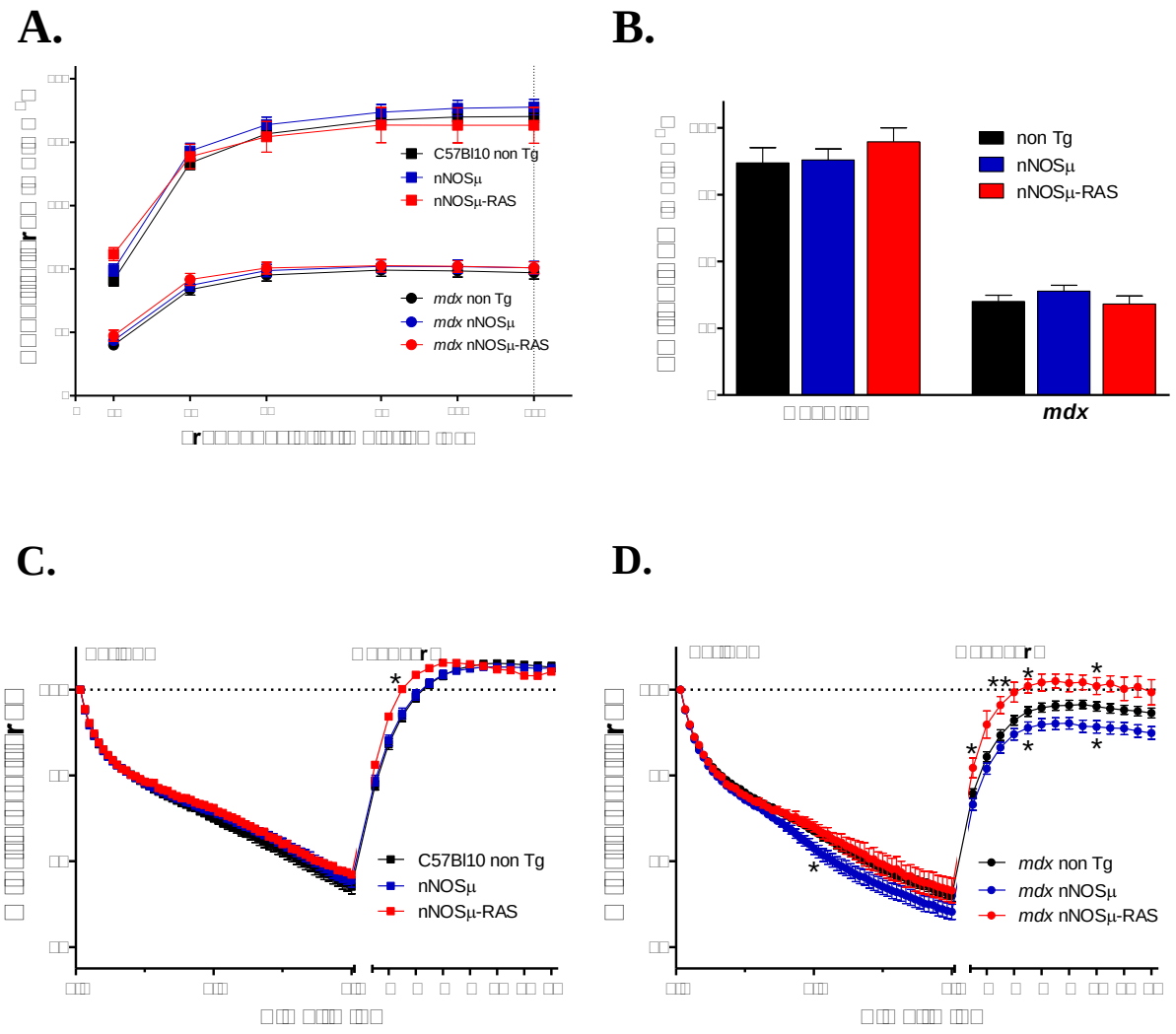


Figure 7. *Ex vivo* evaluation of Diaphragm Performance

Diaphragm strips were used to evaluate muscle function in transgenic mice expressing cytosolic nNOSμ and sarcolemmal localized nNOSμ-RAS. **A.** SpF vs Frequency of stimulation curve. C57Bl10 non Tg n=15, nNOSμ n=11, nNOSμ-RAS n=4. *mdx* non Tg n=15, nNOSμ n=17, nNOSμ-RAS n=5. **B.** Twitch SpF, SpF evoked for a single stimulus, was measured in diaphragm. **C-D**. Diaphragm strips were subjected to a fatigue protocol, consisting of isometric tetanic contractions (120Hz) every 1 second for a total of 1 minute. Fatigue recovery was then measured every 1 minute. Force values (mean ± SEM) are shown as percentage of initial force. **C.** Pooled data for mice in C57Bl10 background. Non Tg n=14, nNOSμ n=14, nNOSμ-RAS n=4. **D.** Pooled data for mice in the *mdx* background. Non Tg n=15, nNOSμ n=12, nNOSμ-RAS n=6. * $P \leq 0.05$; ** $P \leq 0.01$ compared to non Tg control.

agree with previous reports from the lab showing than nNOSμ deficiency has a major impact on increasing muscle fatigue susceptibility (Percival et al., 2008; Percival et al., 2010).

3.2 In situ evaluation of Tibialis Anterior Performance

We performed *in situ* physiology analysis of TA muscle (Percival et al., 2008), allowing for measurement of contractile properties without removing the muscle from its natural environment, maintaining normal vasoregulation and innervation. Briefly, mice were anesthetized with avertin, the TA muscle was surgically isolated and the tendon attached with surgical silk to the force transducer arm, while 2 electrodes were place on the sciatic nerve. The mice was kept asleep with small doses of avertin every 15-20 minutes, while rest on a heated (37°C) platform. We performed Length vs. Tension curve to determine L_0. Then, the SpF was measured at frequencies of 10, 30, 50, 80, 100, 150 and 200Hz, and SpF vs Frequency was plotted comparing control and transgenic lines. SpF was calculated from both left and right hind limbs of each mouse. One hind limb was used to perform the fatigue resistance protocol, while the second hind limb was used to perform ECC-induced injury (explained below), alternating right and left extremities between experiments.

Expressions of nNOSμ have not effect on TA SpF in C57Bl10 or *mdx* mice (Figure 8A). Similarly, TA SpF is not significativaly different when nNOSμ-RAS is expressed. However, slightly higher forces in both, C57Bl10 and *mdx* background, are observed when stimulated at low frequencies (Figure 8A). Then, we measured the TA twitch specific force (Figure 8B). Expression of nNOSμ does not affect the twitch SpF in both C57Bl10 and *mdx* TA. However, nNOSμ-RAS expression tends to increase twitch SpF in normal and dystrophic

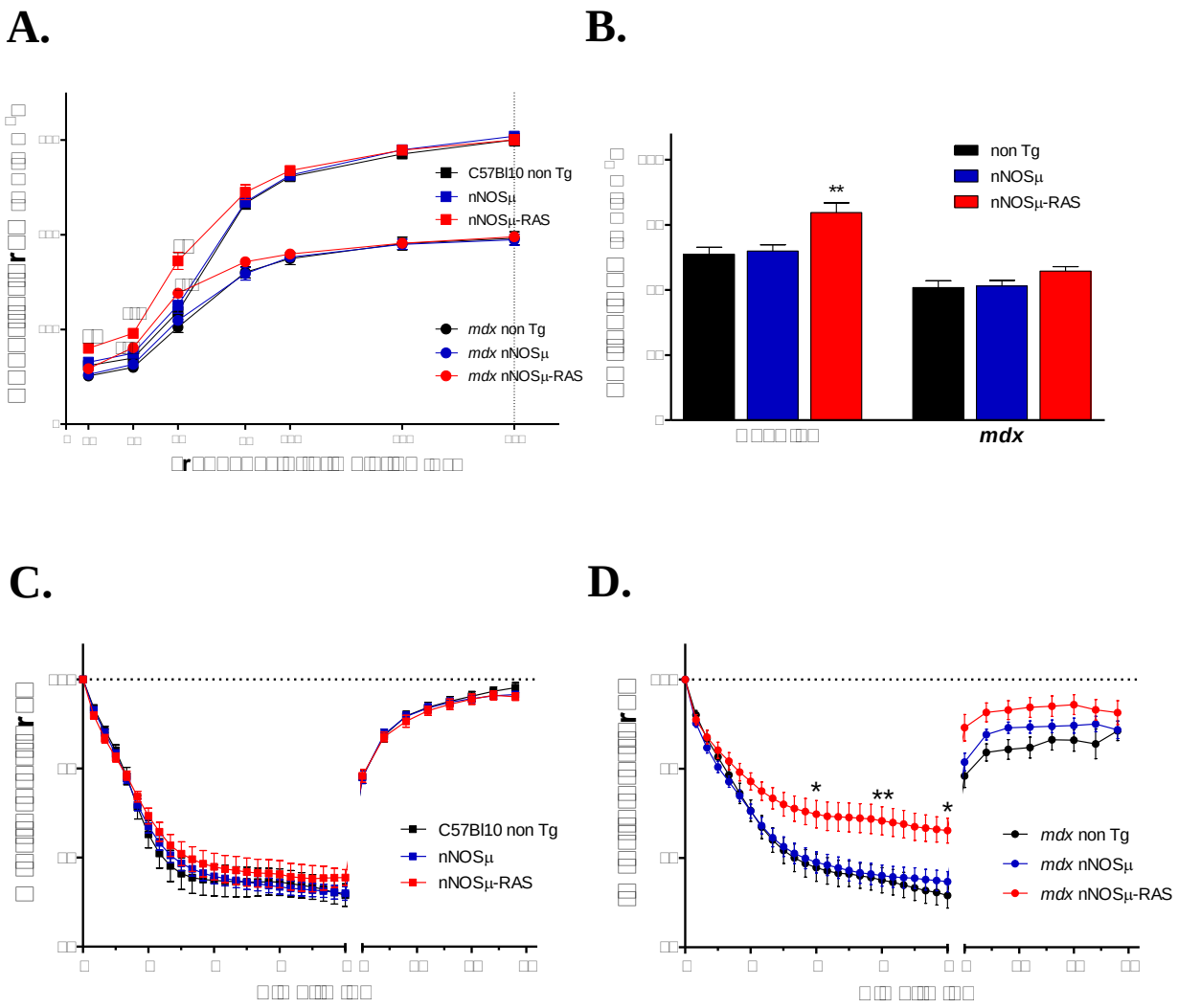


Figure 8. *In situ* evaluation of Tibialis anterior Performance

Whole TA muscle in is natural environment, keeping mouse alive while the experiment was performed, was used to evaluate muscle function in transgenic mice expressing cytosolic nNOSμ and sarcolemmal localized nNOSμ-RAS. **A.** SpF vs Frequency of stimulation curve. C57Bl10 non Tg n=25, nNOSμ n=31, nNOSμ-RAS n=13. *mdx* non Tg n=24, nNOSμ n=32, nNOSμ-RAS n=12. **B.** Twitch SpF in TA. **C-D**. TA was subjected to a fatigue protocol consisting of isometric tetanic contractions (200Hz) every 2 second for a total of 4 minutes. Only values every 10 seconds are shown for clarity. Fatigue recovery was then measured every 2 minutes. Force values (mean ± SEM) are shown as percentage of initial force. **C.** Pooled data for mice in C57Bl10 background. Non Tg n=15, nNOSμ n=17, nNOSμ-RAS n=7. **D.** Pooled data for mice in the *mdx* background. Non Tg n=14, nNOSμ n=17, nNOSμ-RAS n=6. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ compared to non Tg control.

TA, although only in *C57Bl10* the effect is statistically significative. This is consistent with the observation of slightly higher forces when stimulated at low frequencies.

For fatigue experiments, TA muscle was subjected to tetanic stimulation (200Hz) every 2 seconds during 4 minutes (120 contractions) to induce fatigue. After the last contraction, a tetanic stimulation every 2 minutes for at least 15 minutes was performed to evaluate TA muscle recovery. We observed that transgenic mice expressing nNOSμ in the cytosol or sarcolemmal localized nNOSμ show same TA fatigue and recovery pattern than control C57Bl10 mice (Figure 8C). When analyzing dystrophic TA from mice in the *mdx* background, nNOSμ transgenic mice do not present significative differences with regular *mdx* mice; however, nNOSμ-RAS expression leads to significatively less fatigue than *mdx* (Figure 8D).

3.3 Twitch kinetics in Dystrophic Muscle

We evaluated a possible mechanism for the fatigue resistance in skeletal muscle from *mdx* nNOSμ-RAS expressing mice. Less susceptibility to fatigue could be due, between other factors, to increased numbers of slow twitch fibers and fewer fast twitch fibers. Different kinds of muscle fibers have different properties. Fast twitch fibers have a more glycolytic metabolism and thus fatigue more easily than slow twitch myofibers. For example, fibers expressing type I myosin heavy chain (MyHC) are the most fatigue resistant, while those expressing type IIB MyHC are the least fatigue resistant (Larsson and Phillips, 1998; Larsson et al., 1991; Schiaffino and Reggiani, 1994; Schiaffino and Reggiani, 2011) (Figure 9A). It has been shown that muscle fatigue susceptibility can be modulated by changes in the ratio of fatigue-resistant to fatigue susceptible muscle fibers (Percival et al., 2010; Selsby et al., 2012;

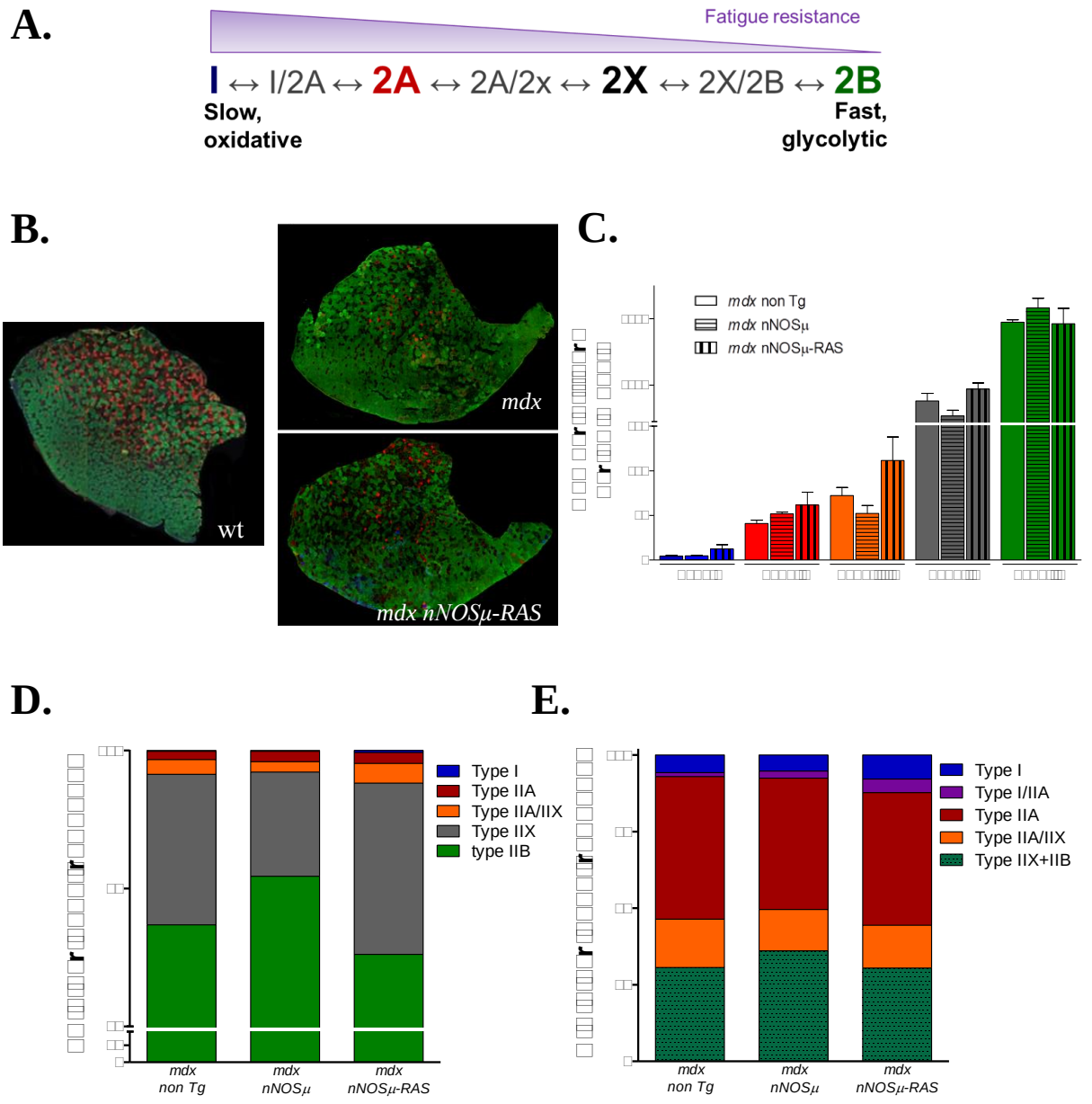


Figure 9. Fiber Typing in Dystrophic Muscle

A. Scheme of fiber types featured in skeletal muscle, their main metabolism and fatigue resistance. **B**. TA muscles where immunostained using antibodies against myosin type I, IIA and IIB to evaluate different fiber types. Colored fibers correspond to the fiber type describe in the code in the scheme. **C.** The number of each fiber type was counted on whole TA from *mdx* mice, *mdx* expressing nNOSμ and *mdx* expressing nNOSμ-RAS (n=3 each). **D.** Frequency of fiber types in TA muscle. **C**. Frequency of fiber types in Diaphragm muscle, evaluated similarly to TA, but on ⅓ of the whole diaphragm from *mdx* mice (n=3), *mdx* expressing nNOSμ (n=4) and *mdx* expressing nNOSμ-RAS (n=4).

Summermatter et al., 2012). A higher proportion of slow twitch fibers would increase the time for the muscle to reach peak twitch tension (TPT) and/or the relaxation phase, measured as the time to reach the half of the relaxation (HRT). Analyzing Twitch kinetics parameters we observed that TPT and HRT were invariable in Diaphragm from nNOSμ expressing mice when comparing with regular *mdx* (Table 2). Conversely, we observed increments of both TPT and HRT in *mdx* expressing nNOSμ-RAS. The same observation was made for Twitch kinetics in TA muscle. This result suggests a possible change in the fiber composition of the DIA and TA muscle as a mechanism for the decreased fatigability of muscles from transgenic mice expressing sarcolemmal nNOSμ. In order to evaluate this possibility, we immunostained 10 μm sections of isopentane-frozen TA and diaphragm muscle using antibodies against myosin type I, IIA and IIB to evaluate different fiber types. Use of fluorophore-conjugated secondary antibodies allowed to visualized colored fibers corresponding to different fiber types (Figure 9B). When wt TA is observed, a high proportion of red type IIA fibers are seen, which are lost on *mdx* mice. When analyzing TA from nNOSμ-RAS mice, we observed an increased proportion of red type IIA fibers. Then the number of each fiber type was counted on whole TA and ⅓ of the whole diaphragm from *mdx*, *mdx* expressing nNOSμ and *mdx* expressing nNOSμ-RAS mice. We analized total number (TA) or frequency (TA and diaphragm) of fiber expressing different myosins. We were able to observe a higher proportion of less fatigable fibers in muscles from nNOSμ-RAS expressing mice (Figure 9C-E). Although this is a modest change, given the very high presence of type IIX and type IIB fibers, this can account in some degree to the decreased fatigability of sarcolemmal nNOSμ expressing mice.

Table 2: Twitch kinetics in Dystrophic muscle

		***mdx* non Tg**	***mdx* nNOSμ**	***mdx* nNOSμ-RAS**
DIA	**TPT**	37.14 ± 1.12	37.7 ± 0.88	47.46 ± 0.85 ***
	HRT	47.97 ± 0.53	37.42 ± 1.34	39.5 ± 1.64 ***
TA	**TPT**	20.51 ± 0.47	20.17 ± 0.29	22.81 ± 0.49 **
	HRT	10.48 ± 0.35	10.25 ± 0.31	14.54 ± 0.8 **

3.4 In situ evaluation of Eccentric Contraction-induced damage in TA muscle.

We used the same *in situ* methodology used for TA fatigue susceptibility experiments to evaluate Eccentric Contraction-induced damage in *mdx* mice. Eccentric contraction (ECC) is the elongation of the muscle during a contraction. It allows muscle fibers to reach higher levels of tension, above levels reached in isometric (no length change) contraction (Blaauw et al., 2010). Usually occurs when an external force exceeds that produced by the muscle and the muscle lengthens, producing negative work (LaStayo et al., 2003). When repetitive, ECC can lead to muscle damage, characterized by structural and ultrastructural alterations, decreased force and pain (LaStayo et al., 2003; Proske and Morgan, 2001). Dystrophic muscles are more susceptible to damage induced by ECC, and can be detected after a few repetitions. This has been demonstrated in dystrophin null murine model (*mdx*) as well in others like α_2-laminin and γ-sarcoglycan-deficient mice (Blaauw et al., 2010). Thus, augmented ECC-induced damage is a common pathological mark, which can be evaluated in our model. We used a protocol that simulates ECC in TA muscle. We did not evaluate this parameter in Diaphragm, because this muscle does not experience ECC normally. Each contraction is the result of a tetanic stimulus (200Hz) in which during the first portion, the muscle executes an isometric contraction. Then, the muscle is stretched to a new length that is 20% longer than L_0, at a velocity of 2 L_0 per second, and the new length is held until the end of the stimulus. The stretching of the muscle causes a decrease in the maximal isometric force of the following contraction. A round of 10 consecutives eccentric contractions was performed every 1 minute. (Figure 10A). The percentage of change between consecutive contractions was then compared between different mice. After 10 ECC, wt C57Bl10 mice presents just a 25% percent of force reduction (Figure 10B), which after a few minutes is

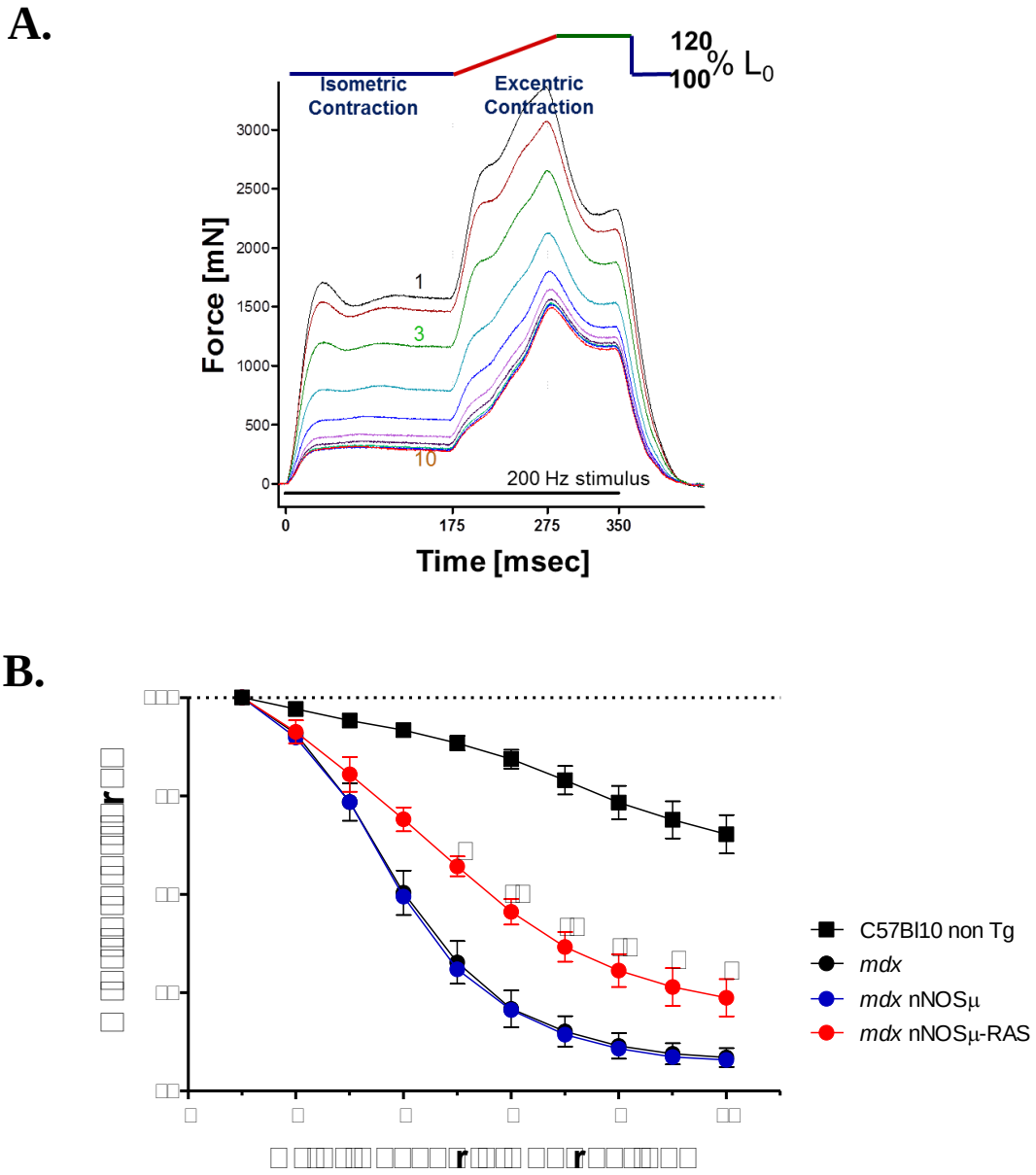


Figure 10. *In situ* evaluation of ECC-induced damage

A. Scheme of ECC protocol. A tetanic stimulus (200Hz) of 350ms of duration was performed. First, muscle executes an isometric contraction. Then, muscle stretches 20% its L_0, at a velocity of 2 L_0 per second, where it holds until the end of the stimulus. Consecutive stretching causes a decrease in the maximal force of the following contraction. 10 consecutives ECC are performed every 1 minute. **B**. TA muscles underwent 10 Eccentric contractions *in situ* and maximal isometric force was measured during each contraction. Pooled data (mean ± SEM) to compare C57Bl10 (n=8), *mdx* (n=15) and *mdx* expressing nNOSμ (n=14) and nNOSμ-RAS (n=7) mice.

almost totally recovered. On the other hand, *mdx* mice present exacerbated ECC-induced damage, decreasing the force to a 44% after 5 ECC, and to around 25% at the end of the protocol. Interestingly, we observed a reduction in the damage caused by ECC in TA muscle from *mdx* expressing sarcolemmal localized NOSμ-RAS, when compared to regular *mdx* mice (Figure 10B). In contrast, expression of nNOSμ showed no improvement.

4. Evaluation of Muscle Pathological Markers

Although the principal aim of this work was characterize the physiological properties of nNOSμ transgenic mice, we also evaluated some typical pathological markers of dystrophic muscle presents in our *mdx* model. The main purpose was looking for some clues of what can be the mechanisms through what nNOSμ-RAS can be decreasing muscle fatigability and ECC-induced damage when expressed in *mdx* mice.

4.1 Centronucleation and fiber area variation.

Fibers from normal muscle appear with regular peripheral nuclei. Upon a damage, muscle experiment necrosis and then regeneration. When in the regeneration process, regenerating fibers appear with central nuclei, which after a few days recover the peripheral localization. On the same context, regenerating fibers appear smaller, getting a normal size at the end of the regeneration process (Cabral et al., 2008; Karalaki et al., 2009). In DMD and several other myopathies, chronic damage leads to ongoing and probably incomplete cycles of degeneration/regeneration, and the percentage of central nuclei rises. However, no consensus

exists about the true meaning of centronucleation and its changes. While some investigators associate increased centronucleation with higher muscle damage, giving to it a negative meaning, it is also true that centronucleation is a sign of regeneration and appearance of new fibers. If the damage of the muscle remains constant, increasing regeneration can help to muscle function, which is our position.

We evaluated the effect of cytosolic and sarcolemmal localized nNOSμ on muscle fiber regeneration through the determination of centronucleated in *mdx* and *mdx* transgenic mice. We used Wheat Germ Aglutinin (WGA)-488 and DAPI to label membrane and nuclei respectively (Figure 11A). This fluorescent staining allows a clear view and easier quantification of centronucleai and fiber area that classical staining with H&E. We evaluated centronucleation in Diaphragm and TA muscles from the fluorescents images obtained, using Image J Cell counter plugin. We did not find differences in centronucleation in diaphragm from *mdx* nNOSμ and *mdx* nNOSμ-RAS transgenic mice when compared with regular *mdx* (Figure 11B). However, we found significative increase of centronuclei in TA muscle from sarcolemmal expressed nNOSμ compared to regular *mdx* and *mdx* with cytosolic expression of nNOSμ (Figure 11C).

Together, because regenerating fibers appear to have smaller caliber, we evaluated TA fiber size (cross-sectional area) distribution, to determine the presence of smaller, probably regenerating fibers. We measured 800-1000 fibers per muscle, distributed on the whole TA, to perform a cumulative frequency graph of fiber cross area. We found that TA fiber area distribution for cytosolic nNOSμ transgenic in the *mdx* background is very similar to regular *mdx*. On the other hand, expression of sarcolemmal localized nNOSμ has a different distribution, showing a higher proportion of smaller fibers (Figue 11D). These results together

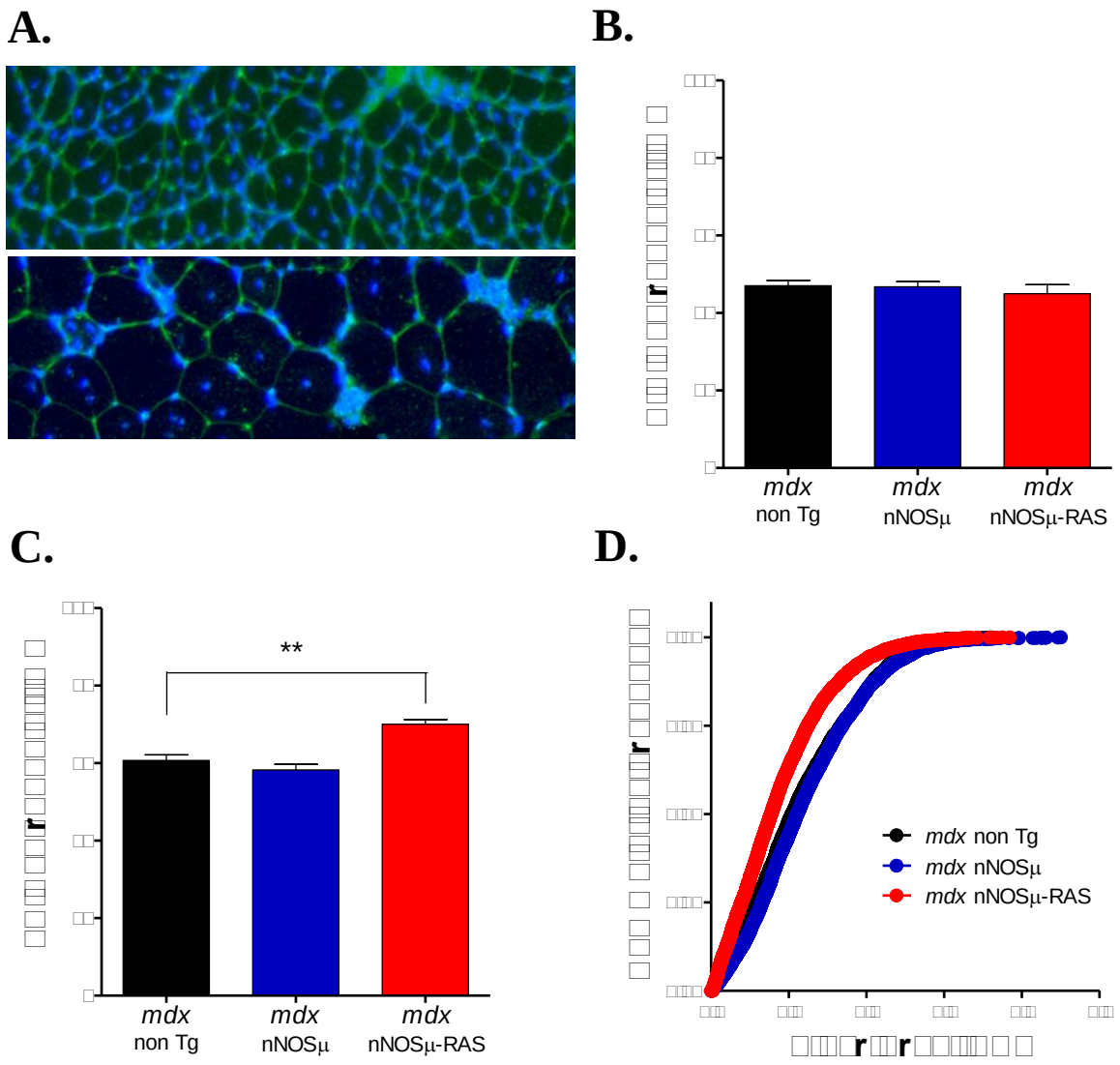


Figure 11. ***Centronucleation and fiber area variation*** **in Dystrophic Muscle**

A. Fluorescent images showing WGA-488 and DAPI staining to observe fiber membrane and nuclei in diaphragm (top) and TA (bottom). Half of the diaphragm or whole TA where analyzed, using a grid to evaluate fibers covering the full area stained. **B**. Percentage of centronuclei in diaphragm from *mdx* (n=10) and transgenic mice in the *mdx* background: nNOSμ (n=15), nNOSμ-RAS (n=5). **C**. Percentage of centronuclei in TA from *mdx* (n=13) and transgenic mice in the *mdx* background: nNOSμ (n=19), nNOSμ-RAS (n=5). Values are mean ± SEM. 1 way Anova with Bonferroni multicomparison test: ** $p \leq 0.01$. **D**. Cumulative Frequency of TA fiber area values for *mdx* (n=5) and *mdx* transgenic mice: nNOSμ (n=9), nNOSμ-RAS (n=5).

suggest that transgenic mice expressing sarcolemal localized nNOSμ present higher levels of regeneration in hind limb muscles. Former observations already have indicated that nNOS and NO may affect muscle differentiation and regeneration (Anderson, 2000; Lee et al., 1994; Wehling et al., 2001). However, our data dissent from those on Wheling et al, 2001, where the expression of cytosolic nNOSα can increase regeneration, as our cytosolic nNOSμ transgenic mice do not present this difference. Whether expression levels or the use of the skeletal muscle (μ) nNOS instead of the brain (α) isoform are making the difference, it remains to be addressed.

4.2 Fibrotic markers evaluation

As mentioned before, DMD and its *mdx* mouse model are also characterized by fibrosis: an excessive accumulation of ECM components such as collagen and fibronectin (Serrano et al., 2011; Serrano and Munoz-Canoves, 2010; Wynn, 2008) that replaces the functional tissue, decreasing muscle force and normal regeneration. Previous work had shown possible that NO signaling can have some effects on fibrosis. Sildenafil is a selective inhibitor of phosphodiesterase (PDE) 5 that allows maintaining active the cGMP-dependent pathway of NO signaling. Administration of sildenafil to *mdx* mice via the drinking water and posterior examination of skeletal muscle function, showed that chronic, long-term treatment significantly reduced *mdx* diaphragm muscle weakness and slowed the establishment of *mdx* diaphragm fibrosis (Percival et al., 2012). Then, we evaluated if some fibrosis markers were affected by nNOSμ or nNOSμ-RAS expression. We used 10-μm-thick frozen sections to perform immunofluorescence with antibodies against fibronectin and collagen I and analyze the accumulation of both ECM components. Analyzing diaphragm, we found no differences in

the area occupied by fibronectin and collagen I between muscles from *mdx* and both transgenic mice (Figures 12A). However, evaluation of the amount of protein measured by WB show that fibronectin is increased in diaphragm from transgenic mice expressing nNOSμ, compared to regular *mdx,* while no differences are found in nNOSμ-RAS expressing muscle. (Figures 12B). When analyzing TA, again we found no differences in these fibrotic markers using immunofluorescence between *mdx* and both transgenic mice. (Figure 13A). WB indicates that, similarly to that seen in diaphragm, cytosolic nNOSμ increases fibronectin accumulation in TA, although not statistically significative (Figure 13B). Our results suggest that expression of nNOSμ-RAS have none effect on fibrosis in the *mdx* background, and that cytosolic expression of cytosolic nNOSμ increases fibronectin accumulation, at least at the age we tested. We used 2 month old mice for muscle physiology characterization, and muscle slides and homogenates were obtained from the same mice. Fibronectin and collagen deposition increases with time and pathology progression in *mdx*, and it is possible that different or more noticeable effects of transgenic expression of cytosolic or sarcolemmal nNOSμ on fibrosis can be seen only later in the pathology of the *mdx* mice.

5. nNOSμ ability to direct DGC members to the sarcolemma

DMD and is caused by mutations in the dystrophin gene, which encodes an actin binding protein that links the actin cytoskeleton with the extracellular matrix, forming the dystrophin-associated glycoprotein complex (DGC) (Figure 2). The dystrophin deficiency leads to loss of other DGC members, including nNOSμ (Ibraghimov-Beskrovnaya et al., 1992; Waite et al., 2009).nNOSμ interacts with α-syntrophin through its PDZ domain, connecting nNOSμ to dystrophin and dystrobrevin, then to the rest of the complex (see Figure 2). Because

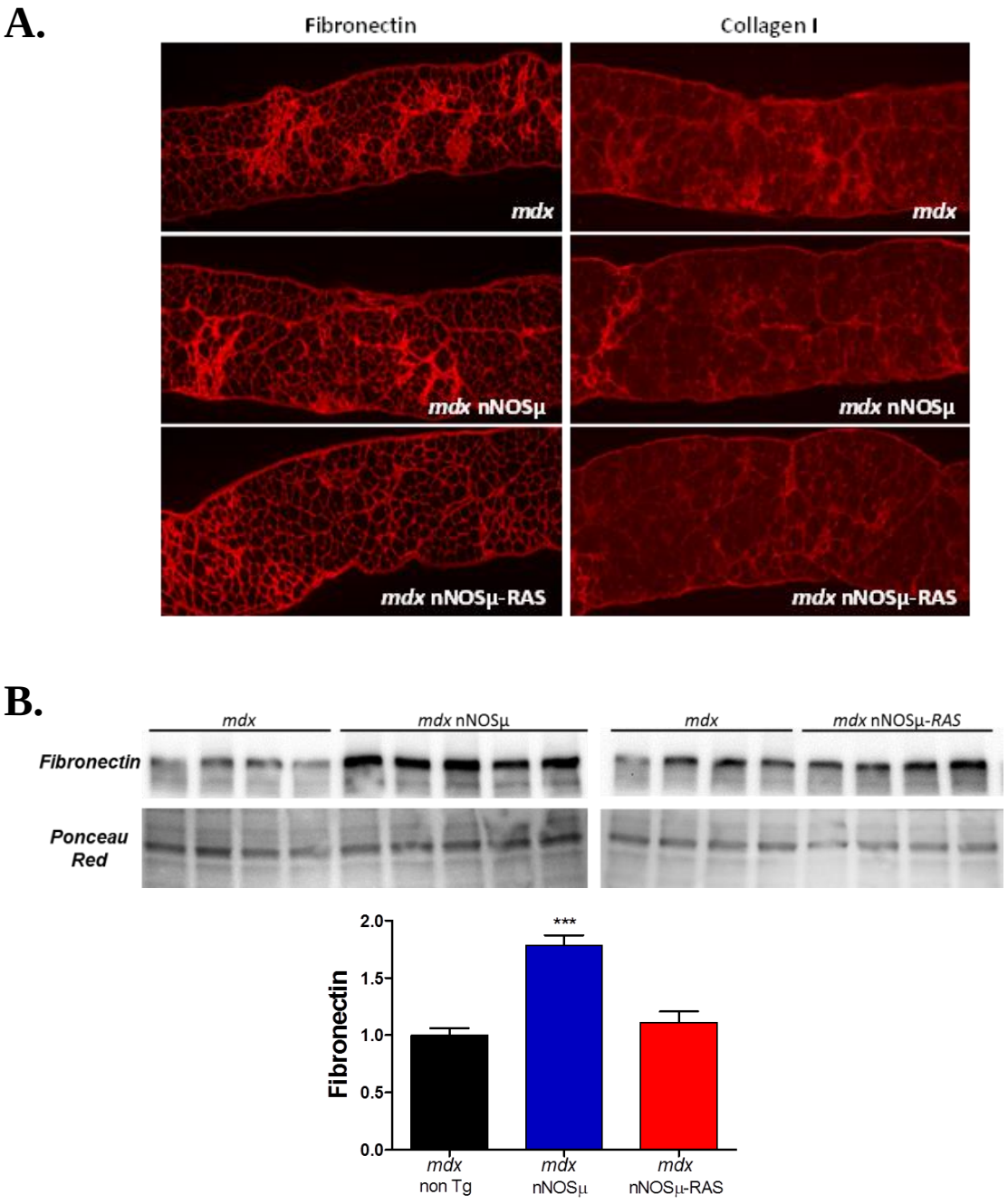


Figure 12. Fibronectin and Collagen I staining in Diaphragm

A. Immunofluorescence for Fibronectin (left) and Collagen I (right). Just a portion of the diaphragm is shown. **B**. Western blot against Fibronectin on Diaphragm homogenates. Transgenic mice expressing nNOSμ and nNOSμ-RAS are compared to non-transgenic littermates. Fibronectin increases in *mdx* mice expressing cytosolic nNOSμ.

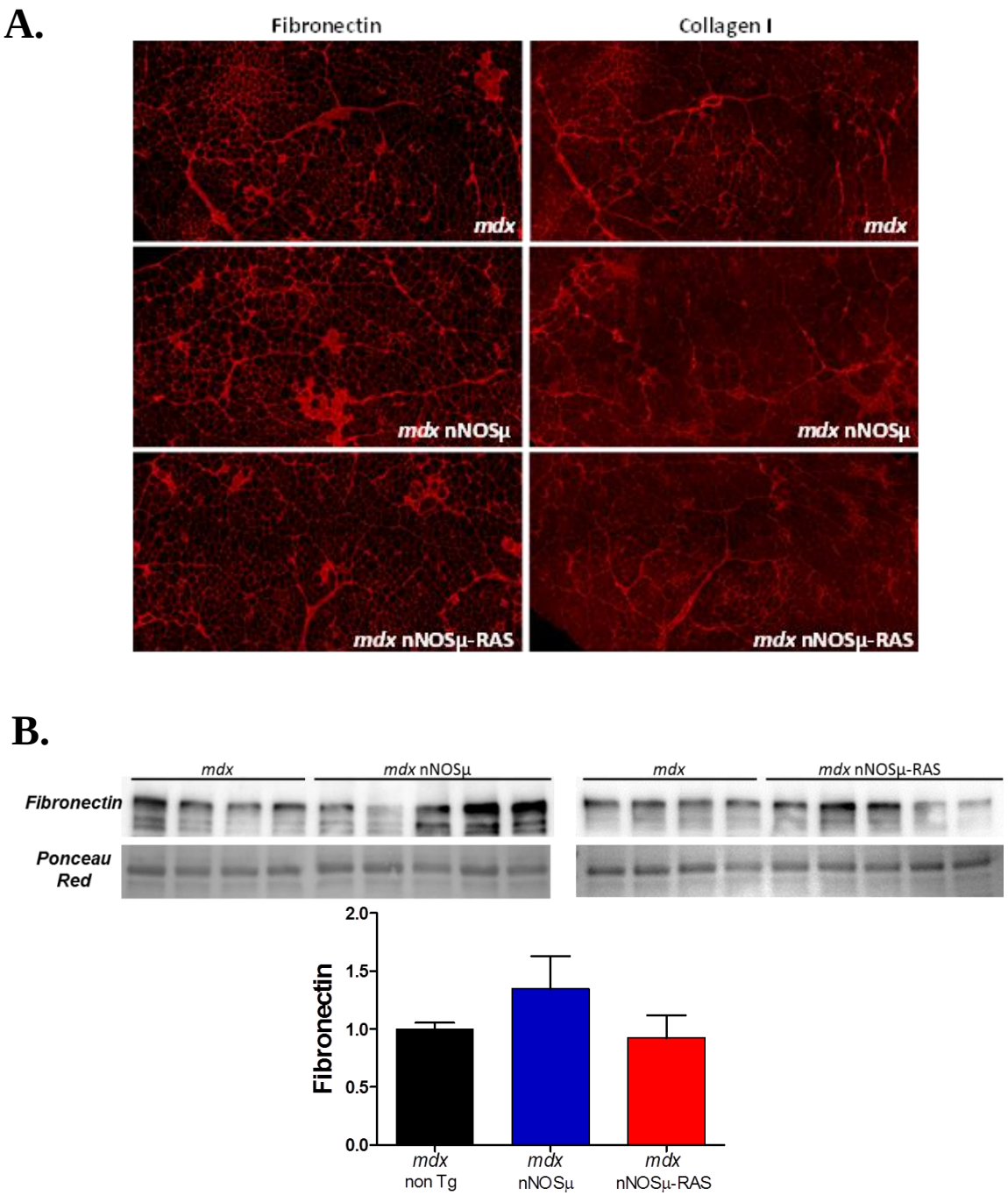


Figure 13. Fibronectin and Collagen I staining in TA muscle

A. Immunofluorescence for Fibronectin (left) and Collagen I (right). Just a portion of the TA is shown, but whole TA was quantified. **B**. Western blot against Fibronectin on TA homogenates. Transgenic mice expressing nNOSμ and nNOSμ-RAS are compared to non-transgenic littermates. Fibronectin increases in *mdx* mice expressing cytosolic nNOSμ.

of one of the roles of DGC is associated to help to maintain membrane integrity, and perhaps the most important result of this work was the reduction in the damage caused by eccentric contractions in the *mdx* nNOSμ-RAS TA muscle, we thought of evaluating whether nNOSμ-RAS transgenic protein is able to restore the sarcolemmal localization of other members of the complex. Because of technical difficulties, we could not evaluate syntrophin localization. Then, we performed a double immunofluorescence against the HA-tag and dystrobrevin. We did not observed sarcolemmal localization of nNOSμ or dystrobrevin on transgenic mice expressing nNOSμ. However, in sarcolemmal localized nNOSμ, we were able to detect dystrobrevin colocalizing with nNOSμ-RAS on the sarcolemma (Figure 14), localization driven most probably trough PDZ interaction with α-syntrophin . It remains to be determined if other components of the complex can also be recluted by nNOSμ-RAS, and if this phenomena is one of the reasons because the nNOSμ-RAS transgenic mouse is more resistant to ECC-induced damage.

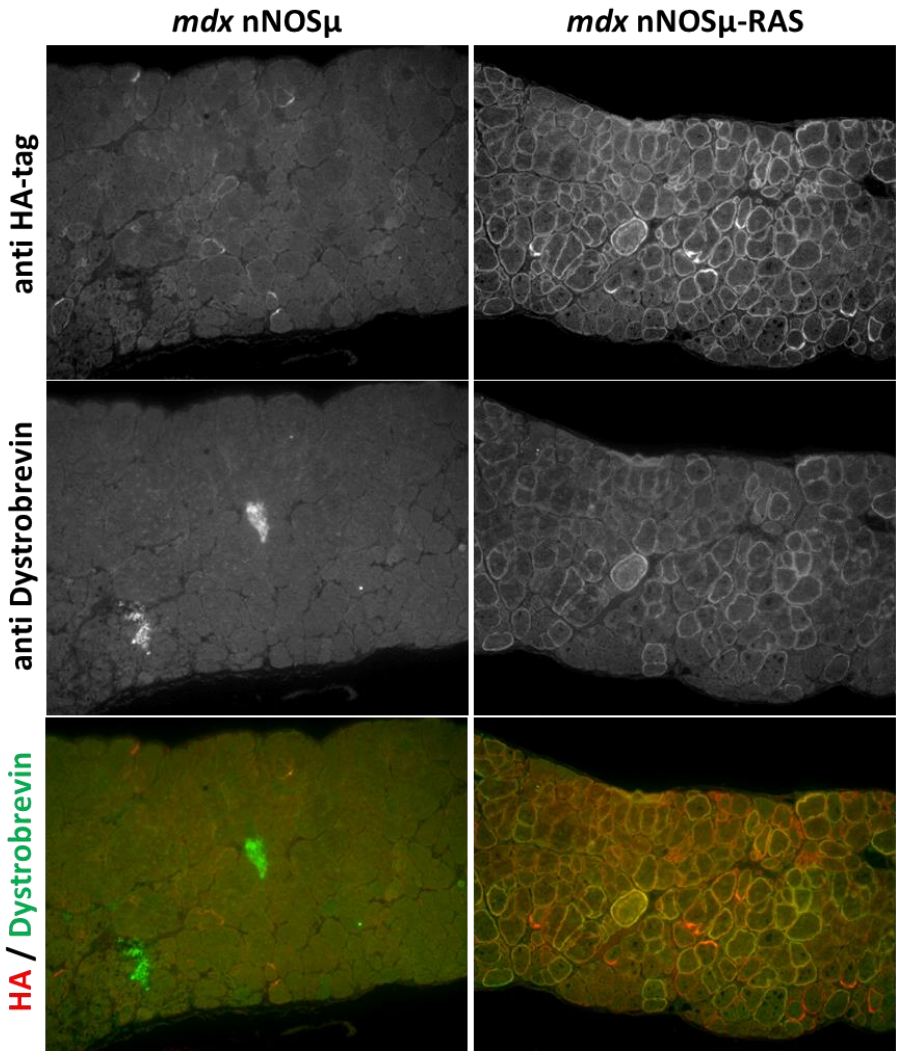


Figure 14. nNOSμ ability to direct DGC members to the sarcolemma

A. Double immunofluorescence against the HA-tag and the DGC member dystrobrevin. nNOSμ localizes to the sarcolemma in the nNOSμ-RAS. Dystrobrevin was detected colocalizing with nNOSμ-RAS on the sarcolemma.

IV. DISCUSSION

Abnormally high levels of NO, especially at subcellular locations where the levels are usually low, can have major detrimental effects. We reasoned that targeting of nNOSμ to the sarcolemma, its normal location, is an essential requirement for improving the dystrophic phenotype. Production of NO at the sarcolemma would increase the likelihood that the appropriate physiological targets would be hit, while at the same time reducing NO mediated damage caused by abnormally high levels of cytosolic nNOSμ. As described above, in this work we have taken a different and novel approach to achieve nNOSμ expression and localization in skeletal muscle. First, for the first time we produced transgenic mice expressing nNOSμ, the skeletal muscle expressed isoform. Previous publications have used the brain nNOSα isoform, which lack of 34 aminoacids characteristics of the μ-domain, whose function remains undetermined. Second, we performed a modification adding the RAS sequence at the C-terminus, resulting in palmitoylation of nNOSμ, a modification that drives the protein to the sarcolemma independent of dystrophin and α-syntrophin presence. Although we describe nNOSμ-RAS localization as sarcolemmal, it is important to indicate that probably the transgenic protein is directed to general plasma membrane, including T-tubules, and also to some internal membranes or vesicles that are in transit to the plasma membrane.

Although generation of the transgenic mice was a long and complex process, we successfully obtain transgenic lines for both nNOSμ and nNOSμ-RAS transgenes. We aimed to functionally characterize the muscle performance of the new transgenic mice. Then, we have conducted extensive functional studies to compare the impact of sarcolemmal nNOSμ-RAS with cytosolic nNOSμ in skeletal muscle.

In wild type mice, nNOSμ can normally localize to the sarcolemma because of the intact DGC complex. Then, ectopic nNOSμ may contribute to physiological functions of normal muscle. It is known that nNOSμ expression and activity increases with age in mice and rats at least until 1 year of age (Chang et al., 1996; Stamler and Meissner, 2001). Furthermore, it is also known that nNOSμ expression is increased with exercise in both rodents and humans, which has been related to a role of nNOSμ in adaptation of skeletal muscle to endurance exercise (Percival, 2011). Modifying nNOSμ expression with the ectopic transgene could mimic the effects of exercise training in wild type muscles. In this context, we expected to observe some increments on muscle force or fatigue resistance when increasing nNOSμ (or nNOSμ-RAS), speeding processes that improve muscle performance. We observed a modest but significative increment in TA SpF when stimulated at low frequencies, but not a change in muscle fatigability. We expected a stronger improved phenotype which we did not observe. Maybe gradual increments of the protein are required later for this purpose, but not early in the embryonic muscle development, where starts to be active the HSA promotor.

We focused on functional studies to evaluate in dystrophic skeletal muscle the impact of sarcolemmal nNOSμ-RAS and cytosolic nNOSμ. Perhaps the most important result was the reduction in the damage caused by eccentric contractions in the *mdx* nNOSμ-RAS TA muscle, a common pathological marker of DMD and the *mdx* model. Earlier studies have shown that nNOSμ absence has a major impact on increasing muscle fatigue susceptibility in normal, non-dystrophic muscle (Percival et al., 2008; Percival et al., 2010). Using an *in situ* protocol, we have now examined the effect of sarcolemmal targeted nNOSμ on muscle fatigue. Fatigue in the *mdx* TA muscle was radically reduced by RAS-nNOSμ on the sarcolemma. Fatigue susceptibility was also reduced when analyzing diaphragm. We did not

observe significative changes in fibrosis, suggesting that the mechanism of action is not trough the diminution of fibrotic markers at the age tested (2 month old), and studies in older mice must be performed to evaluate if restoring sarcolemmal nNOSμ can decrease fibrosis.

About the possible mechanisms improving fatigue and ECC-induced damage in our transgenics, we will discuss some possible factors which posteriorly need to be studied in depth. We observed a slower twitch kinetic in muscles from nNOSμ-RAS expressing mice. These results suggest that fiber type shift can be one of the mechanisms. Following this reasoning, we performed fiber typing in TA and diaphragm. When wt TA is observed, a high proportion of red type IIA fibers are seen, which are lost on *mdx* mice. This phenotype have been also observed in mice lacking nNOSμ (Percival et al., 2010). In *mdx* muscle expressing sarcolemmal localized nNOSμ we observed a subtle higher proportion of slow fibers. This result suggests that nNOSμ in the membrane, but not cytosolic nNOSμ, is preventing the loss of fibers that are more resistant to fatigue. Then, maintenance of less fatigable fibers can be one of the mechanisms, but certainly not the only one, in decreasing fatigue susceptibility in *mdx* mice expressing nNOSμ-RAS.

On the other hand, sarcolemmal localized nNOSμ is essential opposing to vasoconstriction during exercise through NO production and maintaining blood flow and oxygen availability (Thomas et al., 1998; Thomas et al., 2003). In muscular dystrophies as DMD, DGC loss includes delocalization and decreased expression of nNOSμ, while nNOSμ loss from the sarcolemma is also observed in models of denervation and ALS (Brenman et al., 1995; Chao et al., 1996; Crosbie et al., 2002; Meinen et al., 2012; Percival, 2011; Suzuki et al., 2010; Suzuki et al., 2007). This common phenomenon, added to the reduced vascularization in muscular dystrophy (Gargioli et al., 2008), can trigger ischemic foci and a

detrimental hypoxic environment. Restoring nNOSμ to the sarcolemma would help to maintain oxygen availability during muscle contraction, having enough energy production for muscle contraction, reducing fatigue, and preventing necrotic foci because of hypoxia.

We also presented some evidence suggesting that expression of nNOSμ localized in the sarcolemma can promote regeneration in dystrophic TA muscle. Previously has been reported that nNOSμ inhibition or its absence (*mdx* or nNOS knockout) negatively affect satellite cell activation, including morphological hypertrophy and decreased adhesion in the fiber-lamina complex (Anderson, 2000). In addition, it has been shown how the use of NO donors can increase myogenesis and muscle repair (De Palma and Clementi, 2012; Stamler and Meissner, 2001). Although these reports do not empathize on the localization of NO production and literature indicates that both cytosolic and sarcolemmal nNOSμ are important in muscle function, our results suggest that in *mdx* mice expression of sarcolemmal but not cytosolic nNOSμ is able to induce regeneration. Actions of NO on skeletal muscle mentioned above, such as vasodilation and thus reduction of the ischemia induced by nNOSμ displacement, increase in glucose uptake and its angiogenic effects (Stamler and Meissner, 2001), all of them facilitated if NO production is on the sarcolemma, may also have contributed to muscle repair, and to reduced ECC-induced damage and Fatigue susceptibility in our transgenics (Schgoer et al., 2009; Ziche et al., 1994).

The role of cytoplasmic nNOSμ has become a controversial issue. In the nNOSμ/dystrophin double knockout mouse, the loss of nNOSμ results in an increase in the strength of the EDL and TA muscles (Li et al., 2011). This indicates that cytosolic nNOSμ may have a toxic function in dystrophic muscle, a function already suggested for non-dystrophic muscles: α1-syntrophin-null muscles show displacement of nNOSμ from the

sarcolemma and do not regenerate normally (Hosaka et al., 2002), while in tail-suspension, denervation and ALS models, nNOSμ has been shown to be misslocalized and induce muscle atrophy through the activation of Foxo3a and muscle-specific E3 ubiquitin ligases MuRF-1 and atrogin-1/MAFbx (Suzuki et al., 2010; Suzuki et al., 2007).Taking this in count, improvement on muscle performance in nNOSμ-RAS expressing mice can also be due, at least in part, to a reduction of the toxic effects of cytosolic endogenous nNOSμ. nNOS form active dimers (Zhou and Zhu, 2009), therefore monomeric nNOSμ with the RAS-tag could associate to endogenous nNOSμ and recruit it to the sarcolemma in the absence of dystrophin. Here, an additive action between restoration of normal function and diminution of toxic function can account for muscle performance improvement in nNOSμ-RAS transgenic mice. Nevertheless, we did not observed a generalized worsening of muscle function in transgenic mice expressing cytosolic nNOSμ. While no effects were observed on TA muscles, a slight increment in fatigue susceptibility and fibronectin accumulation was observed in Diaphragm from nNOSμ transgenic mice. If the gain of toxic function of nNOSμ is precise, we expected to observe a much severe worsen phenotype in *mdx* mice. However, different mechanisms in dystrophic muscle can be acting since early muscle development to attempt protecting muscle cells from this harm, mechanisms that are not presents in other models where nNOSμ displacement from the sarcolemma is induced in adult mice in an acute manner.

Finally, we observed recruitment of the DGC-member dystrobrevin to the sarcolemma of nNOSμ-RAS expressing mice. Although this observation was coincidental, due to use of dystrobrevin to detect revertant fibers conjunctly to nNOSμ, it was gratefully to detect that nNOSμ-RAS transgene have the ability to recruit other DGC members to the sarcolemma. First, because the recruitment of dystrobrevin can lead to the recruitment of other

DGC members, as sarcoglycans and surely α-syntrophin. DGC proteins are important for establishing the physical connection between the ECM and the cytoskeleton and play a role in transmitting force related to muscle contraction (Allikian and McNally, 2007; Waite et al., 2009). Thus, although not recovering the whole complex, partial restoration of DGC members can help to maintain membrane integrity and interaction with different signaling pathways that are lost in the absence of dystrophin, making a milder phenotype. Second, because this observation supports our hypothesis that nNOSμ-RAS could associate to endogenous nNOSμ and recruit it to the sarcolemma, diminishing toxic function of cytosolic endogenous nNOSμ.

It is important to mention that this work does not resolve the question whether NO production or nNOSμ scaffold function are the responsible for the phenotype observed in nNOSμ-RAS expressing skeletal muscle. Although, we think a combination of both activities are producing the effects observed. Knowing the real NO amounts generated by transgenic enzyme results difficult as all activity assays do not discriminate between nNOS, (endothelial) e NOS or (inducible) iNOS NO production. nNOS produces NO at low (pico to nanomolar range) physiological levels for short periods. Conversely, iNOS is independent of the Ca^{2+}/calmodulin complex and generates NO at high concentrations (micromolar range) for prolonged periods (De Palma and Clementi, 2012). Then, changes in nNOSμ-derived NO production result masked by NO generated by the very high levels of iNOS present in the *mdx* mice, which is developing an important and chronic inflammatory response. On the other hand, nNOSμ is not only a signaling enzyme, but has a scaffold function that is becoming more important of what originally was thought. Interaction with α-syntrophin and through it to all DGC is well described, but it remains unknown if the μ-domain, the 34 amino acids not present in the brain isoform nNOSα, can interact and maybe serve as a scaffold to other

proteins which can have a role in muscle function. Furthermore, we have shown that the DGC member dystrobrevin can be localized to the sarcolemma when nNOSμ is on the plasma membrane, even if dystrophin is absent. It remains to be determined if other components of the complex can also be recluted by nNOSμ-RAS. However, this evidence strongly suggests that not only chemical reaction but structural function of nNOSμ are important for muscle function. We propose that using of specific, or partially specific, NOS inhibitors as well as the transgenic expression of a nNOSμ-RAS transgenic protein without catalytic activity can help to dissect whether NO production or nNOSμ scaffold properties are determinant for the effects observed in our transgenic mice.

V. FUTURE DIRECTIONS

We developed for the first time transgenic mice expressing nNOSμ muscle specific isoform. Future experiments derived from this work include the evaluation of the role of the alternatively spliced isoform. The function of the μ-domain is unknown, but has been postulated to be involved in membrane association and possible posttranslational regulation of nNOSμ (Larsson and Phillips, 1998; Silvagno et al., 1996). Interaction with proteins, different or in addition to those interacting with nNOSα, could also be another form of particular regulation for nNOSμ.

nNOSμ expression, localization and/or signaling are impaired in many neuromuscular diseases of diverse genetic etiology, including Duchenne muscular dystrophy (DMD) (Brenman et al., 1995), BMD(Chao et al., 1996), LGMD 2C, 2D and 2E (Crosbie et al., 2002), Ullrich congenital muscular dystrophy, inflammatory myositis (Kobayashi et al., 2008), muscle atrophy by denervation and ALS (Suzuki et al., 2010; Suzuki et al., 2007). Thus, independent sarcolemmal localization of nNOSμ is a powerful instrument to evaluate nNOSμ signaling in different pathologic scenarios. Accordingly, DMD and the *mdx* model was our scenario of impaired nNOSμ signaling where to study nNOSμ function. From here, a big list of future experiments using this transgenic line is being established. First example is the evaluation of restoration of nNOSμ in different models of muscular dystrophies and other neuromuscular diseases where nNOSμ signaling is defective. So far, it has been proposed to study mice lacking γ-sarcoglycan (gsg-/- mice), a model of human LGMD2C; these mice have severe skeletal muscle pathology (Hack et al., 1998), more representative of the human disease, and significantly decreased survival (~50% death in 5 months). Also, it has been proposed the evaluation of sarcolemmal nNOSμ restoration in mice with sciatic denervation

and ALS models, in the context of hypoxic response as a consequence of nNOSμ displacement and the effects on muscular fibrosis. Moreover, with the use of nNOSμ-RAS expression it has been proposed determine which defects in skeletal muscle of the KN2 mouse (which lacks both nNOSμ and nNOSβ, the two nNOS isoforms ex*p*ressed in skeletal muscle) can be corrected by nNOSμ alone. The KN2 mouse has a very severe phenotype (Gyurko et al., 2002). The muscle phenotype of the KN1 mouse, which lacks only nNOSμ, is much less severe (Percival et al., 2008; Percival et al., 2010), suggesting that nNOSβ serves an important function, even though it is expressed at only low levels (about 10% of nNOSμ). Restoration of nNOSμ will likely produce new information about the relative roles of the sarcolemmal and Golgi forms of nNOS in skeletal muscle.

In our transgenic studies, RAS-nNOSμ expression is driven by the HSA-promoter, which is active early in embryonic muscle development. To have human therapeutic value, phenotypic improvement of nNOSμ-RAS expression after diagnosis will be required. Thus, another future perspective from this work is using AAV6 muscle-specific expression system, an effective and thoroughly characterized method for viral delivery to adult mouse and canine skeletal muscle (Gregorevic et al., 2006; Judge and Chamberlain, 2005; Wang et al., 2012), to be used in the study of nNOSμ function and evaluation of therapeutic potential in models of muscular dystrophy.

VI. REFERENCES

Allikian, M.J., and E.M. McNally. 2007. Processing and assembly of the dystrophin glycoprotein complex. *Traffic*. 8:177-183.

Anderson, J.E. 2000. A role for nitric oxide in muscle repair: nitric oxide-mediated activation of muscle satellite cells. *Mol Biol Cell*. 11:1859-1874.

Bender, A.T., and J.A. Beavo. 2006. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev*. 58:488-520.

Blaauw, B., L. Agatea, L. Toniolo, M. Canato, M. Quarta, K.A. Dyar, D. Danieli-Betto, R. Betto, S. Schiaffino, and C. Reggiani. 2010. Eccentric contractions lead to myofibrillar dysfunction in muscular dystrophy. *J Appl Physiol*. 108:105-111.

Brenman, J.E., D.S. Chao, S.H. Gee, A.W. McGee, S.E. Craven, D.R. Santillano, Z. Wu, F. Huang, H. Xia, M.F. Peters, S.C. Froehner, and D.S. Bredt. 1996. Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. *Cell*. 84:757-767.

Brenman, J.E., D.S. Chao, H. Xia, K. Aldape, and D.S. Bredt. 1995. Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell*. 82:743-752.

Cabral, A.J.V., V. Machado, R. Farinha, and A. Cabrita. 2008. Skeletal muscle regeneration: a brief review

António J. V. Cabral1, Vanessa Machado1, Rodrigo Farinha1, António Cabrita1. *Experimental Pathology and Health Sciences2008; 2 (2): 9-17*. 2:9.

Chang, W.J., S.T. Iannaccone, K.S. Lau, B.S. Masters, T.J. McCabe, K. McMillan, R.C. Padre, M.J. Spencer, J.G. Tidball, and J.T. Stull. 1996. Neuronal nitric oxide synthase

and dystrophin-deficient muscular dystrophy. *Proc Natl Acad Sci U S A*. 93:9142-9147.

Chao, D.S., J.R. Gorospe, J.E. Brenman, J.A. Rafael, M.F. Peters, S.C. Froehner, E.P. Hoffman, J.S. Chamberlain, and D.S. Bredt. 1996. Selective loss of sarcolemmal nitric oxide synthase in Becker muscular dystrophy. *J Exp Med*. 184:609-618.

Craven, K.B., and W.N. Zagotta. 2006. CNG and HCN channels: two peas, one pod. *Annu Rev Physiol*. 68:375-401.

Crawford, G.E., J.A. Faulkner, R.H. Crosbie, K.P. Campbell, S.C. Froehner, and J.S. Chamberlain. 2000. Assembly of the dystrophin-associated protein complex does not require the dystrophin COOH-terminal domain. *J Cell Biol*. 150:1399-1410.

Crosbie, R.H., R. Barresi, and K.P. Campbell. 2002. Loss of sarcolemma nNOS in sarcoglycan-deficient muscle. *Faseb J*. 16:1786-1791.

De Palma, C., and E. Clementi. 2012. Nitric oxide in myogenesis and therapeutic muscle repair. *Mol Neurobiol*. 46:682-692.

Durbeej, M., and K.P. Campbell. 2002. Muscular dystrophies involving the dystrophin-glycoprotein complex: an overview of current mouse models. *Curr Opin Genet Dev*. 12:349-361.

Ehmsen, J., E. Poon, and K. Davies. 2002. The dystrophin-associated protein complex. *J Cell Sci*. 115:2801-2803.

Eliasson, M.J., S. Blackshaw, M.J. Schell, and S.H. Snyder. 1997. Neuronal nitric oxide synthase alternatively spliced forms: prominent functional localizations in the brain. *Proc Natl Acad Sci U S A*. 94:3396-3401.

Eu, J.P., J.M. Hare, D.T. Hess, M. Skaf, J. Sun, I. Cardenas-Navina, Q.A. Sun, M. Dewhirst, G. Meissner, and J.S. Stamler. 2003. Concerted regulation of skeletal muscle contractility by oxygen tension and endogenous nitric oxide. *Proc Natl Acad Sci U S A*. 100:15229-15234.

Eu, J.P., J. Sun, L. Xu, J.S. Stamler, and G. Meissner. 2000. The skeletal muscle calcium release channel: coupled O2 sensor and NO signaling functions. *Cell*. 102:499-509.

Gargioli, C., M. Coletta, F. De Grandis, S.M. Cannata, and G. Cossu. 2008. PlGF-MMP-9-expressing cells restore microcirculation and efficacy of cell therapy in aged dystrophic muscle. *Nat Med*. 14:973-978.

Gregorevic, P., J.M. Allen, E. Minami, M.J. Blankinship, M. Haraguchi, L. Meuse, E. Finn, M.E. Adams, S.C. Froehner, C.E. Murry, and J.S. Chamberlain. 2006. rAAV6-microdystrophin preserves muscle function and extends lifespan in severely dystrophic mice. *Nat Med*. 12:787-789.

Gyurko, R., S. Leupen, and P.L. Huang. 2002. Deletion of exon 6 of the neuronal nitric oxide synthase gene in mice results in hypogonadism and infertility. *Endocrinology*. 143:2767-2774.

Hack, A.A., C.T. Ly, F. Jiang, C.J. Clendenin, K.S. Sigrist, R.L. Wollmann, and E.M. McNally. 1998. Gamma-sarcoglycan deficiency leads to muscle membrane defects and apoptosis independent of dystrophin. *J Cell Biol*. 142:1279-1287.

Hakim, C.H., D. Li, and D. Duan. 2011. Monitoring murine skeletal muscle function for muscle gene therapy. *Methods Mol Biol*. 709:75-89.

Hofmann, F., D. Bernhard, R. Lukowski, and P. Weinmeister. 2009. cGMP regulated protein kinases (cGK). *Handb Exp Pharmacol*:137-162.

Hosaka, Y., T. Yokota, Y. Miyagoe-Suzuki, K. Yuasa, M. Imamura, R. Matsuda, T. Ikemoto, S. Kameya, and S. Takeda. 2002. Alpha1-syntrophin-deficient skeletal muscle exhibits hypertrophy and aberrant formation of neuromuscular junctions during regeneration. *J Cell Biol*. 158:1097-1107.

Ibraghimov-Beskrovnaya, O., J.M. Ervasti, C.J. Leveille, C.A. Slaughter, S.W. Sernett, and K.P. Campbell. 1992. Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature*. 355:696-702.

Judge, L.M., and J.S. Chamberlain. 2005. Gene therapy for Duchenne muscular dystrophy: AAV leads the way. *Acta Myol*. 24:184-193.

Karalaki, M., S. Fili, A. Philippou, and M. Koutsilieris. 2009. Muscle regeneration: cellular and molecular events. *In Vivo*. 23:779-796.

Kobayashi, Y.M., E.P. Rader, R.W. Crawford, N.K. Iyengar, D.R. Thedens, J.A. Faulkner, S.V. Parikh, R.M. Weiss, J.S. Chamberlain, S.A. Moore, and K.P. Campbell. 2008. Sarcolemma-localized nNOS is required to maintain activity after mild exercise. *Nature*. 456:511-515.

Larsson, B., and S.C. Phillips. 1998. Isolation and characterization of a novel, human neuronal nitric oxide synthase cDNA. *Biochem Biophys Res Commun*. 251:898-902.

Larsson, L., L. Edstrom, B. Lindegren, L. Gorza, and S. Schiaffino. 1991. MHC composition and enzyme-histochemical and physiological properties of a novel fast-twitch motor unit type. *Am J Physiol*. 261:C93-101.

LaStayo, P.C., J.M. Woolf, M.D. Lewek, L. Snyder-Mackler, T. Reich, and S.L. Lindstedt. 2003. Eccentric muscle contractions: their contribution to injury, prevention, rehabilitation, and sport. *J Orthop Sports Phys Ther*. 33:557-571.

Lee, K.H., M.Y. Baek, K.Y. Moon, W.K. Song, C.H. Chung, D.B. Ha, and M.S. Kang. 1994. Nitric oxide as a messenger molecule for myoblast fusion. *J Biol Chem*. 269:14371-14374.

Li, D., Y. Yue, Y. Lai, C.H. Hakim, and D. Duan. 2011. Nitrosative stress elicited by nNOSmicro delocalization inhibits muscle force in dystrophin-null mice. *J Pathol*. 223:88-98.

Meinen, S., S. Lin, M.A. Ruegg, and A.R. Punga. 2012. Fatigue and muscle atrophy in a mouse model of myasthenia gravis is paralleled by loss of sarcolemmal nNOS. *PLoS One*. 7:e44148.

Miyagoe-Suzuki, Y., and S.I. Takeda. 2001. Association of neuronal nitric oxide synthase (nNOS) with alpha1-syntrophin at the sarcolemma. *Microsc Res Tech*. 55:164-170.

Percival, J.M. 2011. nNOS regulation of skeletal muscle fatigue and exercise performance. *Biophysical Reviews*. 3:9.

Percival, J.M., K.N. Anderson, P. Gregorevic, J.S. Chamberlain, and S.C. Froehner. 2008. Functional deficits in nNOSmu-deficient skeletal muscle: myopathy in nNOS knockout mice. *PLoS One*. 3:e3387.

Percival, J.M., K.N. Anderson, P. Huang, M.E. Adams, and S.C. Froehner. 2010. Golgi and sarcolemmal neuronal NOS differentially regulate contraction-induced fatigue and vasoconstriction in exercising mouse skeletal muscle. *J Clin Invest*. 120:816-826.

Percival, J.M., N.P. Whitehead, M.E. Adams, C.M. Adamo, J.A. Beavo, and S.C. Froehner. 2012. Sildenafil reduces respiratory muscle weakness and fibrosis in the mdx mouse model of Duchenne muscular dystrophy. *J Pathol*. 228:77-87.

Proske, U., and D.L. Morgan. 2001. Muscle damage from eccentric exercise: mechanism, mechanical signs, adaptation and clinical applications. *J Physiol*. 537:333-345.

Rando, T.A. 2001. The dystrophin-glycoprotein complex, cellular signaling, and the regulation of cell survival in the muscular dystrophies. *Muscle Nerve*. 24:1575-1594.

Ross, R.M., G.D. Wadley, M.G. Clark, S. Rattigan, and G.K. McConell. 2007. Local nitric oxide synthase inhibition reduces skeletal muscle glucose uptake but not capillary blood flow during in situ muscle contraction in rats. *Diabetes*. 56:2885-2892.

Sambrook, J., P. MacCallum, and D. Russell. 2001. Molecular Clonig: A Laboratory Manual.

Schgoer, W., M. Theurl, J. Jeschke, A.G. Beer, K. Albrecht, R. Gander, S. Rong, D. Vasiljevic, M. Egger, A.M. Wolf, S. Frauscher, B. Koller, I. Tancevski, J.R. Patsch, P. Schratzberger, H. Piza-Katzer, A. Ritsch, F.H. Bahlmann, R. Fischer-Colbrie, D. Wolf, and R. Kirchmair. 2009. Gene therapy with the angiogenic cytokine secretoneurin induces therapeutic angiogenesis by a nitric oxide-dependent mechanism. *Circ Res*. 105:994-1002.

Schiaffino, S., and C. Reggiani. 1994. Myosin isoforms in mammalian skeletal muscle. *J Appl Physiol*. 77:493-501.

Schiaffino, S., and C. Reggiani. 2011. Fiber types in mammalian skeletal muscles. *Physiol Rev*. 91:1447-1531.

Schillings, M.L., J.S. Kalkman, H.M. Janssen, B.G. van Engelen, G. Bleijenberg, and M.J. Zwarts. 2007. Experienced and physiological fatigue in neuromuscular disorders. *Clin Neurophysiol*. 118:292-300.

Selsby, J.T., K.J. Morine, K. Pendrak, E.R. Barton, and H.L. Sweeney. 2012. Rescue of dystrophic skeletal muscle by PGC-1alpha involves a fast to slow fiber type shift in the mdx mouse. *PLoS One*. 7:e30063.

Serrano, A.L., C.J. Mann, B. Vidal, E. Ardite, E. Perdiguero, and P. Munoz-Canoves. 2011. Cellular and molecular mechanisms regulating fibrosis in skeletal muscle repair and disease. *Curr Top Dev Biol*. 96:167-201.

Serrano, A.L., and P. Munoz-Canoves. 2010. Regulation and dysregulation of fibrosis in skeletal muscle. *Exp Cell Res*. 316:3050-3058.

Shiao, T., A. Fond, B. Deng, M. Wehling-Henricks, M.E. Adams, S.C. Froehner, and J.G. Tidball. 2004. Defects in neuromuscular junction structure in dystrophic muscle are corrected by expression of a NOS transgene in dystrophin-deficient muscles, but not in muscles lacking alpha- and beta1-syntrophins. *Hum Mol Genet*. 13:1873-1884.

Silvagno, F., H. Xia, and D.S. Bredt. 1996. Neuronal nitric-oxide synthase-mu, an alternatively spliced isoform expressed in differentiated skeletal muscle. *J Biol Chem*. 271:11204-11208.

Stamler, J.S., and G. Meissner. 2001. Physiology of nitric oxide in skeletal muscle. *Physiol Rev*. 81:209-237.

Stedman, H.H., H.L. Sweeney, J.B. Shrager, H.C. Maguire, R.A. Panettieri, B. Petrof, M. Narusawa, J.M. Leferovich, J.T. Sladky, and A.M. Kelly. 1991. The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. *Nature*. 352:536-539.

Summermatter, S., R. Thurnheer, G. Santos, B. Mosca, O. Baum, S. Treves, H. Hoppeler, F. Zorzato, and C. Handschin. 2012. Remodeling of calcium handling in skeletal muscle

through PGC-1alpha: impact on force, fatigability, and fiber type. *Am J Physiol Cell Physiol*. 302:C88-99.

Suzuki, N., H. Mizuno, H. Warita, S. Takeda, Y. Itoyama, and M. Aoki. 2010. Neuronal NOS is dislocated during muscle atrophy in amyotrophic lateral sclerosis. *J Neurol Sci*. 294:95-101.

Suzuki, N., N. Motohashi, A. Uezumi, S. Fukada, T. Yoshimura, Y. Itoyama, M. Aoki, Y. Miyagoe-Suzuki, and S. Takeda. 2007. NO production results in suspension-induced muscle atrophy through dislocation of neuronal NOS. *J Clin Invest*. 117:2468-2476.

Thomas, G.D., M. Sander, K.S. Lau, P.L. Huang, J.T. Stull, and R.G. Victor. 1998. Impaired metabolic modulation of alpha-adrenergic vasoconstriction in dystrophin-deficient skeletal muscle. *Proc Natl Acad Sci U S A*. 95:15090-15095.

Thomas, G.D., P.W. Shaul, I.S. Yuhanna, S.C. Froehner, and M.E. Adams. 2003. Vasomodulation by skeletal muscle-derived nitric oxide requires alpha-syntrophin-mediated sarcolemmal localization of neuronal Nitric oxide synthase. *Circ Res*. 92:554-560.

Tidball, J.G., and M. Wehling-Henricks. 2004. Expression of a NOS transgene in dystrophin-deficient muscle reduces muscle membrane damage without increasing the expression of membrane-associated cytoskeletal proteins. *Mol Genet Metab*. 82:312-320.

Waite, A., C.L. Tinsley, M. Locke, and D.J. Blake. 2009. The neurobiology of the dystrophin-associated glycoprotein complex. *Ann Med*. 41:344-359.

Wang, Y., D.C. Newton, and P.A. Marsden. 1999. Neuronal NOS: gene structure, mRNA diversity, and functional relevance. *Crit Rev Neurobiol*. 13:21-43.

Wang, Z., R. Storb, C.L. Halbert, G.B. Banks, T.M. Butts, E.E. Finn, J.M. Allen, A.D. Miller, J.S. Chamberlain, and S.J. Tapscott. 2012. Successful regional delivery and long-term expression of a dystrophin gene in canine muscular dystrophy: a preclinical model for human therapies. *Mol Ther*. 20:1501-1507.

Wehling-Henricks, M., M. Oltmann, C. Rinaldi, K.H. Myung, and J.G. Tidball. 2009. Loss of positive allosteric interactions between neuronal nitric oxide synthase and phosphofructokinase contributes to defects in glycolysis and increased fatigability in muscular dystrophy. *Hum Mol Genet*. 18:3439-3451.

Wehling, M., M.J. Spencer, and J.G. Tidball. 2001. A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice. *J Cell Biol*. 155:123-131.

Whitehead, N.P., C. Pham, O.L. Gervasio, and D.G. Allen. 2008. N-Acetylcysteine ameliorates skeletal muscle pathophysiology in mdx mice. *J Physiol*. 586:2003-2014.

Wynn, T.A. 2008. Cellular and molecular mechanisms of fibrosis. *J Pathol*. 214:199-210.

Zhou, L., and D.Y. Zhu. 2009. Neuronal nitric oxide synthase: structure, subcellular localization, regulation, and clinical implications. *Nitric Oxide*. 20:223-230.

Ziche, M., L. Morbidelli, E. Masini, S. Amerini, H.J. Granger, C.A. Maggi, P. Geppetti, and F. Ledda. 1994. Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. *J Clin Invest*. 94:2036-2044.