Comparative Analysis of the Neuromuscular Junction in Aging Rats and Sarco Mice

Daren Elkrief

Department of Kinesiology and Physical Education

Faculty of Education

McGill University, Montreal, Quebec

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LIST OF ABBREVIATIONS

Symbol	Definition
NMJ	Neuromuscular Junction
AChR	Acetylcholine Receptor
MuSK	Muscle Specific Kinase
Rapsyn	Receptor Associated Protein at the Synapse
VL	Vastus Lateralis
YA	Young Adult
VO	Very Old
GPS	Gastrocnemius-Plantarus-Soleus Complex
SARCO	Sarco Mouse
WT	Wild Type
EPP	End Plate Potential
ALS	Amyotrophic Lateral Sclerosis
F344BN	Fischer 344 Brown x Norway Rat
SOD1	Super Oxide Dismutase 1
TrkB	Tyrosine Receptor Kinase B
ATG7	Autophagy
COURAGE	Collaborative Research on Ageing in Europe
MHC	Myosin Heavy Chain
DMD	Duchenne Muscular Dystrophy

PGC 1α	Proliferator-Activated Receptor Gamma Coactivator 1-alpha
TGFβ	Transforming Growth Factor Beta
FoxO	Forkhead Box O
NF-ϰB	Nuclear Factor Kappa B
MuRF-1	Muscle Ring Finger 1
TEM	Transmission Electron Microscope
CCD	Charge Coupled Device
EM	Electron Microscope
αBTX	αbungarotoxin
pSC	Peripheral Schwann Cell
NT	Nerve Terminal
SMA	Spinal Muscular Atrophy
MRF	Myogenic Regulatory Factors
TA	Tibialis Anterior
MFCV	Muscle Fiber Conduction Velocity
Dok7	Docking Protein 7
GGT	Geranylgeranyl Transferase
APC	Adenomatous Polyposis Coli
MG	Myasthenia Gravis
SCS	Slow Channel Syndrome
Lrp4	Low-density Lipoprotein Receptor-related Protein 4
BDNF	Brain Derived Neurotrophic Factor
NT-3/4	Neurotrophin-3/4

GDNF	Glial Derived Neurotrophic Factor
CNTF	Ciliary Neurotrophic Factor
IGF	Insulin-like Growth Factor
FGF	Fibroblast Growth Factor
EGF	Epidermal Growth Factor
ROS	Reactive Oxygen Species
Akt	Protein Kinase B
mTOR	Muscle Target of Rapamycin
SNAP25	Synaptosome Associated Protein 25 killodalton
UCP1	Uncoupling Protien 1
TDP43	TAR DNA-binding Protein 43
JNK	c-Jun NH(2)-terminal kinase

ABSTRACT

Skeletal muscle atrophy with advancing age can result in loss of independence in the elderly [1]. Amongst the most important contributors to deterioration of aging muscle are changes at the neuromuscular junction (NMJ) that render aging myocytes more susceptible to denervation [2]. In this respect, since fragmentation of the acetylcholine receptor (AChR) clusters in aging muscle occurs well before loss of motor neuron cell bodies can be detected in the spinal cord, much of the current research in aging muscle is focused upon identifying the myocellular mechanisms causing AChR cluster fragmentation at the aging NMJ[3]. Since the agrin-Muscle Specific Kinase (MuSK) pathway is essential for maintenance of the adult NMJ, we hypothesized that this pathway would be perturbed in aging muscle. To address this, we immunolabeled cross-sections of *vastus lateralis* (VL) muscle from young adult (YA) and very old (VO) Fisher 344xBrown Norway F1-hybrid rats for AChR, MuSK, and rapsyn, and compared this to immunolabeled cross-sections of *soleus* (Sol) muscle from wild type (WT) and transgenic neurotrypsin over-expressing mice (Sarco), the latter representing a model of impaired MuSK activation. There was a decrease in the AChR cluster integrity ratio with aging (3.354 ± 0.9770 versus 1.433 ± 0.1495 versus (mean ± SD) in YA vs VO, respectively) as well as in the Sarco mice (0.6471 ± 0.07658 compared to the wild type mice (0.8821 ± 0.03532). In the Sarco mice, there was a 44% decrease in MuSK and 11% decrease in Rapsyn intensity at the NMJ, accompanied by a 25% reduction in AChR intensity, which were expected given the reduced agrin-MuSK signalling. In contrast, although we also observed a 34% decrease in MuSK (down 34%(48.85 ±24.19 AU) in VO versus YA (74.30 ±37.9 AU $p<0.05$)) with aging, there was an increase in Rapsyn (up 18%

(57.65±23.07 AU) in VO versus YA (48.81±17.06 AU $p< 0.05$) and AChR was only reduced modestly (down 10% (91.27 ± 43.03 AU) in VO versus YA (101.6 ±41.35 $p<0.05$). As such, our results suggest that the agrin-MuSK pathway is relatively well-preserved in aging muscle. Finally, to further assess NMJ stability, we calculated the Rapsyn:AChR ratio which showed a large relative increase in the ratio ((up 38% in VO (0.719 ±0.3156) compared to YA (0.5199 ±0.1810)). Rapsyn: AChR intensity also increased in SARCO (0.8442±0.2410 $p<0.05$) compared to WT (0.7325±0.1766 $p<0.05$), but unlike aging it was driven by a reduction in AChR rather than an increase in rapsyn. We conclude that instability of the aging NMJ is likely not related to impaired Agrin-MuSK signalling.

RESUMÉ

L'atrophie musculaire qui survient avec le vieillissement entraine une perte d'autonomie chez les personnes âgées[1]. Un des composantes menant à l'atrophie musculaire serait une altération des jonctions neuromusculaires (NMJ) [2]. La dénervation qui survient avec le vieillissement induit une fragmentation des récepteurs d'acetylcholine (AChR) affectant la transmission de l'influx nerveux. Ce mécanisme accélère le processus de l'atrophie musculaire lors du vieillissement. L'objectif principal de ce mémoire est d'évaluer les mécanismes sous-jacents au changement des NMJs pendant le vieillissement. Nous émettons l'hypothèse que l'affinité du récepteur kinase specific des muscles (MuSK) est diminuée avec le vieillissement. Afin de vérifier notre hypothèse, ce présent mémoire vise à évaluer plusieurs composants morphologiques des NMJs avec un modèle de rongeur et chez des participants jeunes et vieux rats Fischer 344 Brown-Norway (F344BN). Afin d'investiguer les jonctions neuromusculaires, nous avons utilisé plusieurs modèles : le vastus latérale (VL), un muscle squelettique, investigué chez les rats F344BN jeunes et âgés, et aussi le muscle soléaire dans les souris sauvages (WT) et transgeniques, les Sarco mice (SARCO). Les souris Sarco ont un défaut dans la signalization du MuSK. Nos résultats démontrent une diminution dans le ratio de l'intégrité de la morphologie de l'AChR chez les très vieux participants (3,354 ± 0,9770 contre 1,433 ± 0,1495 (moyenne ± SD) dans YA vs VO, respectivement), ainsi que chez les souris Sarco (0,6471 ± 0,07658 contre 0,8821 ± 0,03532, $p<0,05$ chez les souris types sauvage). Dans les souris Sarco, il y a eu une diminution de 44 % de MuSK et une abaissement de 11% de l'intensité de Rapsyn, accompagnée d'une réduction de 25 % dans

l'intensité de l'AChR. Cette dernière est attribuée à la diminution de la signalisation entre agrin et MuSK. Nos résultats démontrent aussi une diminution de 34 % de MuSK (48,85 ± 24,19 UA) dans le groupe VO contre YA (74,30 ± 37,9 AU $p<0,05$)) avec le vieillissement. Il y a aussi eu une augmentation de 18 % de Rapsyn (57,65 ± 23,07 UA) en VO contre YA (48,81 ± 17,06 AU $p<0,05$) et finalement, l'AChR a été légèrement réduit (moins de 10 % (91,27 ± 43,03 UA) en VO contre YA (101,6 ± 41,35 $p<0,05$). Ainsi, nos résultats suggèrent que la signalisation entre l'agrin-MuSK est bien conservée dans les muscles âgés. Enfin, pour mieux évaluer la stabilité de la NMJ, nous avons calculé le ratio de l'intensité de Rapsyn à AChR qui a démontré une grande augmentation (jusqu'à 38 % en VO (0,719 ± 0,3156) comparé à YA (0,5199 ± 0,1810)). L'intensité de Rapsyn:AChR a également augmenté dans les souris Sarco (0,8442 ± 0,2410 $p<0,05$) comparé à WT (0,7325 ± 0,1766 ($p<0,05$)), mais contrairement aux résultats dans les rats, cela peut être expliquer par une réduction de l'AChR plutôt qu'une augmentation de Rapsyn. Nous concluons que l'instabilité de la NMJ avec le vieillissement n'est probablement pas lié a une perte d'affinité des récepteurs Agrin-MuSK.

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PREFACE & CONTRIBUTIONS OF AUTHORS

D.Elkrief[1,2,3] , R.T. Hepple[1,2,3], S. Spendiff[2,3]

[1]Department of Kinesiology and Physical Education, McGill University; Montreal, QC, Canada

[2]Research Institute of the McGill University Health Centre; Montreal, QC, Canada

[3]McGill Research Centre for Physical Activity & Health. McGill University, Montreal, QC, Canada

D. Elkrief was the primary author and completed the data collection, analysis and interpretation and manuscript preparation.

S.Spendiff aided in experiment design, analysis and interpretation, and manuscript editing.

R.T. Hepple was the principle investigator of the study and aided in study design, results interpretation and manuscript preparation.

CHAPTER 1: INTRODUCTION

1.1 Background

The demographic representation of older individuals is increasing at its fastest rate in history. Between 1975 and 2025, the world's proportion of older adults is expected to grow by 200%[4], which will have important ramifications for healthcare in the future. A large part of the increased health burden can be attributed to the physiological changes and comorbidities [5, 6] associated with advanced age, which significantly affect health and quality of life [7-10]. Amongst these changes, the age-dependent loss of muscle mass and strength heavily affect quality of life, disability, and mortality outcomes in a variety of diseases and during aging[11]. Muscle mass accounts for nearly half of the body weight in adults and is considered a critical factor for independent living among the elderly [6]. Therefore the attenuation of age-related muscle atrophy would effectively reduce the burden of multiple diseases among those most vulnerable to disease and disability [12-14]. Beyond regular exercise (which loses its efficacy in advanced age), there are currently no prescribed medical treatments or drugs for age-related muscle atrophy [15], yet there are a few in clinical trails[16]. It is therefore important to determine the mechanisms of age-related muscle atrophy to provide a basis for developing potential treatments. This review will therefore focus on what is known of the molecular mechanisms of age-related muscle atrophy. Increased denervation, decreased reinnervation potential, mitochondrial dysfunction, and impaired second messenger signalling are among the mechanisms that will be explored in this review.

In recent years reports have shown that denervation, wherein the nerve-muscle synapse ceases signal conduction, is in large part responsible for the mass and force loss seen in aging muscle [17, 18]. Normally a motor neuron signals a single fiber to contract

by sending vesicles containing Ach through the synaptic space. AChR in the post-synaptic membrane receive these signals and if enough are received within a given time period, the fiber is stimulated to contract. Denervated fibers can no longer receive input signals from the presynaptic terminal and therefore do not generate an end plate potential (EPP). There are a variety of avenues by which a nerve loses signaling contact with its innervated fiber. A nerve can pull back or retract, and if a myofiber is damaged the myofiber may fall out of acetylcholine (Ach) signaling range. Denervation therefore occurs under various circumstances, including motor neuron pull back[19], motor unit degeneration[20], or by fiber damage[21] . It has also been suggested that unstable NMJs may impair synaptic transmission and have been shown to cause lead to muscle loss, although this hypothesis has not been tested in age. As such, denervated fibers do not contribute to force development during muscle contraction and additionally, prolonged denervation precipitates muscle fiber atrophy[17]. Not only does denervation occur as part of the normal process of aging, it also occurs under many conditions some of which include trauma [22], diabetic neuropathy [23], myasthenia gravis (MG) [24], and amyotrophic lateral sclerosis (ALS) [25]. Throughout most of adult life denervation and reinnervation are in equilibrium, resulting in a plastic, yet zero net change in total innervation [26]. However, denervation rate is thought to eventually outpace reinnervation rate with age [17, 27], and atrophy is thought to accelerate as the length of denervation persists. Meanwhile muscles become more apoptotic[28] without fiber replacement, which further decreases mass and strength [29]. The causes of denervation are complex and the relative importance of each factor, whether they are molecular or cellular, is unknown. Recent studies have focused on the

interface between the motor neuron and muscle fiber, the neuromuscular junction (NMJ), in seeking to understand the causes of age-related denervation [3, 30, 31]. Studies have shown that the NMJ decreases in stability with age[32], yet the factors contributing to NMJ instability with aging remain unclear. One potential point of instability involves the dysregulation of the proteins required for maintenance of the adult NMJ. These include a variety of structural and signalling proteins, such as agrin, muscle specific tyrosine kinase (MuSK) and Receptor-associated protein at the synapse (rapsyn). Neural agrin organizes the acetylcholine receptor (AChR) during development through actin-induced remodelling of lipid domains [33] and simultaneously induces MuSK dimerization and activation of its co-receptor low -density lipoprotein receptor-related protein4 (LRP4) at the post-synaptic membrane [34]. Agrin is most well-known for its involvement in the development of the NMJ and will be explored in more detail in section. Agrin also induces phosphorylation of MuSK, which in turn stimulates AChR clustering through rapsyn, and cytoskeleton binding via adenomatous polyposis coli (APC) [35]. Diseases that target any one of these proteins result in disassembly of the NMJ, as seen in anti – MuSK and anti- AChR myasthenia gravis [36]. Additionally, using agrin antibodies or over-expressing an endogenous protease called neurotrypsin also results in disassembly of the NMJ within days [37, 38]. Considering the role of the agrin-MuSK pathway in development and NMJ maintenance, it is conceivable that this pathway may be perturbed during aging. For this reason, we wished to assess the quantitative and spatial distribution of proteins related to the agrin-MuSK pathway in aging rat and a mouse model of sporadic denervation consequent to reduced MuSK activation (the Sarco mouse). Specifically, we examined a mouse exhibiting overexpression of the endogenous agrin

antagonist neurotrypsin, which decreases the amount of agrin available at the NMJ. Decreased agrin leads to a decrease in MuSK activation (phosphorylation), which decreases rapsyn-AChR interaction and leads to a reduced AChR clustering. Another group has used this mouse as a model of sporadic denervation and has shown that these mice recapitulate many of the phenotypes of wild-type aging muscle [39]. Simultaneous use of the Fischer 344xBrown Norway F1-hybrid (F344BN) rat model and Sarco mouse model allows us to determine which phenotypes in age could potentially originate from impairment in the agrin-MuSK signalling axis. The potential for defects due to denervation to contribute to aging muscle phenotypes have already been established through the use of SOD1 knock out[40], TrkB heterozygous knockout[41] (which has been shown to induce NMJ disassembly[42]) and ATG7 knockout mice[43]. While some of the changes in the aforementioned models have been compared to age, this study is the first to make a direct comparison between the muscle fibers and NMJs of an aging model and a model with a precise post-synaptic NMJ defect. As such, the purpose of our study was to test the hypothesis that changes in the agrin-MuSK pathway contribute to the fragmentation of the AChR clusters as seen in aging. To answer our question, we compared protein levels between young adult (YA) (8 month) and very old (VO) (35 month) F344BN rats. We focused on muscle at very advanced age because it is equivalent to a clinically relevant age in humans[44, 45] and the point at which a significant accumulation of persistently denervated fibers has accrued [27]. We used in-situ labeling of AChRs by α-bungarotoxin as well as antibody-labeled MuSK and rapsyn in cross-sections of the rat *vastus lateralis* and Sarcomouse *soleus* in order to measure protein intensities at the NMJs of aging rats.

CHAPTER 2: LITERATURE REVIEW

2.1 Demographic Aging and Age Related Muscle Atrophy

2.1.1 Population Aging in North America

Age-related muscle loss has been recognized to include the loss of power and physical performance [46, 47]. This review will consider muscle mass loss due solely to age, yet one should appreciate that age-related diseases have their own effects on muscle mass. Among those afflicted, severe muscle loss is recognized to decrease quality of life, increase disability, and increase mortality outcomes in a variety of diseases [11]. As previously mentioned, given that muscle mass accounts for half of the weight in adults, it is not only the most important factor for stability and independent living [6] but the metabolic implications of decreased lean mass also include an impaired thermoregulative capacity [48] and may also contribute to age-associated glucose intolerance [49]. As also previously mentioned, a global reduction in age related muscle atrophy would have an impact on disability as well. While the current world load of disability among the elderly is estimated at 14% [14], this figure is expected to rise as the populace ages and as the proportion of those with severe muscle loss increases. In one study, it was shown that men with low mass for their age were three times more likely to be physically disabled compared to those who fell within a healthy range of muscle [13]. Additionally, a cross sectional study on 18,363 participants (≥ 65 years old) done by Collaborative Research on Ageing in Europe (COURAGE) concluded that the reduction of age-related muscle loss would also reduce the global burden of disability and disease [12].

2.1.2 Prevalence and Impact of Age-related Muscle Atrophy

A review of multiple studies and metastudies on the impact of age-related muscle atrophy showed that depending on the tool used, the prevalence of age-related muscle atrophy could range from 8.4 – 27.6% among all Americans [14]. One extensive survey of the New Mexico elderly, used by the authors as a proxy for the greater American demographic, found a varied distribution among age groups and noted a 13-14% prevalence in those below 70 years of age and greater than 50% prevalence in those older than 80 years of age [50]. This discrepancy was showcased by a study, which analyzed results across 7 different clinical definitions for age-related muscle atrophy. They found that among Danish male participants, age-related muscle atrophy could range from 0-20.8% among 0-60 year olds, 0-31.2% among 60-69 year olds, and 0-45.2% among >70 year olds[51]. A number of factors such as low daily function, low physical activity levels and a high body mass index, increase the chance of age-related muscle atrophy as well as premature death [52]. Beyond the environmental factors, scientists have for a long time held that the most important causes of age-related muscle atrophy are cellular or physiological in nature, some of which include decreased reinnervation potential, mitochondrial dysfunction, and protein imbalance. More specifically a phenomenon known as denervation at the neuromuscular junction (NMJ), wherein a neuron no longer conducts signals through a synapse to a muscle, is thought to be a primary contributor to functional and mass loss in age [17, 29]. Currently there are no drugs administered for severe muscle loss, yet a search of the EU clinical trials register indicates that there are currently 13 treatments in clinical trials, some of which started as early as 2005[16]. Despite recent progress, however, the interventions currently available to the physically

frail are limited. We therefore believe that there is a value in studying the primary molecular mechanisms of age-related muscle atrophy. In the sections that follow, a review of a number of cellular and molecular mechanisms will indicate that denervation is an important component of atrophy.

2.2 Denervation as a Primary Cause of Age Related Muscle Atrophy

2.2.1 Denervation and Reinnervation Cycles

Denervation at the neuromuscular junction was shown by early studies to contribute to muscle loss, with more changes occurring at the nerve terminal rather than the axon, perhaps indicating damage initiated at the nerve terminal as in Myasthenia Gravis [53]. It is also possible that impairment in transport through axon as is suggested in some ALS research[54, 55]. There is some evidence that early NMJ damage is driven by damage in the myocyte [21]. Since its first reporting [56], denervation has been considered by many to be a primary cause of age-related muscle atrophy [17, 29]. Evidence continues to accumulate in support of this claim. There are a number of events shown to be associated with repeated cycles of denervation and reinnervation. Common indicators of denervation include fiber type co-expression [17], fiber type-grouping [27, 57], fiber size heterogeneity [58, 59], and NMJ fragmentation [60, 61]. Understanding the process of denervation is critical to understanding its contribution to age related muscle atrophy.

Denervation can occur as both a sporadic and a long-term process, as a result of trauma or disease. In sporadic denervation, single motor neurons pull back from the NMJ and the fiber can thereafter no longer receive signals from the nerve. Fibers that were

once controlled by those motor neurons cease contributing to force generation. In response to denervation, fibers secrete neurotrophic factors that induce dendrite sprouting from remaining motor neurons and promote reinnervation [62]. Usually, a terminal axon from a single motor unit will reinnervate the same fiber. With age, however, the fidelity of the process decreases and neighboring motor units may reinnervate fibers from a different motor unit [63]. When this occurs, there is a chance that the fiber type shifts completely or becomes a co-expressing fiber [57, 64]. This occurs because the innervating neuron 'type' may not match its recipient fiber type. Additionally, losing innervation may switch expression of a critical determinant of the muscle fiber's 'fiber type', the myosin heavy chain (MHC)[65]. This denervation and reinnervation cycle generally compensates with little force and size loss. Over many cycles of denervation and reinnervation motor units increase in size and decrease in number. A particularly robust motor unit may increase in size as a result of continued reinnervation of failing nearby fibers[66, 67]. Evidence for motor neuron dieback is seen in electrophysiological studies, which demonstrate that increased size and decreased number of motor units with age could be due to compensatory reinnervation. After fiber denervation, axonal sprouting of nearby motor units results in the reinnervation of a fiber. Motor unit growth has been shown to occur in both humans [67-69] and rats [70]. Traditionally, electrophysiological techniques have indicated that motor unit numbers, particularly type II motor units, decrease with age [66, 71, 72]. Another viewpoint is that preferential type I atrophy in age is an over-simplified aspect of the field and should be explored more closely. Given that some slow fibers atrophy to the same or even greater extent than fast fibers[73] and given that severe atrophy of type I fibers in aging rat soleus muscle is

masked by fiber type co-expression [74], we suspect that the canonical preferential age-related type II affect has been overestimated.

2.2.2 Denervation Leads to Mass loss

There are many paths to fiber loss. There is some evidence that early NMJ damage can be driven by damage in the myocyte. Aging in a mouse model suggests that pre- and post- synaptic NMJ damage accumulates from 3 to 29 months of age, yet the motor neuron counts between those times have been shown to be relatively unchanged [3]. Others have suggested that fiber damage accounts for the increased fragmentation of the post-synaptic AChR clusters in age [21], yet motor neurons can remain normal in the face of massive fiber loss [75]. However, inducing a muscle specific SOD1 knockout has been shown to lead to defects in motorneurons[76], and muscle-specific expression od Igf-1 has been shown to both stabilize NMJs and enhance motor neiron survivale in $SOD1^{G93A}$ ALS mice[77], highlighting the multifactorial nature of neuromuscular diseases and age related muscle loss. In light of the complexity of age-related muscle wasting, it is no surprise that there is no consensus on where motor neuron degeneration, NMJ instability and denervation stand in relation to each other. It appears that there may be many avenues leading to phenotypes, which collectively appear as aged and degenerated muscle. Regardless of the mechanism, lifelong motor neuron loss and denervation can result in a 50% loss of limb muscle mass in those over the age of 80 compared to young adults [29]. If denervation persists a fiber will atrophy progressively and result in an increase in small angular fibers [78, 79]. Interestingly, one study showed that the remaining innervated fibers only show a 7% decrease in size compared to young adult, whereas denervated (identified as those exhibiting $Nav_{1.5}$ positive labeling)

senescent fibers showed a 35% average area decrease suggesting that the majority of fiber atrophy with aging is due to denervation [17]. In order to place motor unit loss, NMJ destruction, and atrophy in relation to each other and in the context of denervation, the following sections will outline fiber –specific changes which support the idea that denervation is a primary cause of age-related muscle atrophy.

2.2.3 Evidence of Denervation and Physiological Markers of Age Related Muscle Atrophy: Fiber Type

Denervation has a number of effects on muscle structure that manifest clearly at the fiber level, as well as at the molecular and whole-muscle level. Understanding fiber classification is critical to tracking changes produced by denervation. Fibers are classified by their myosin heavy chain (MHC) isoform as type I, type IIa , type IIx, and in rodents type IIb. Type I fibers, or slow fibers, have high oxidative and low glycolytic potential, meaning that they rely largely on oxidative phosphorylation and have a high mitochondria content. These fibers produce the least amount of peak force, but can sustain force for a long period of time. Type II or fast fibers exist as type IIa and IIx in humans. Type IIa fibers are fast-oxidative, which have both a high oxidative and glycolytic capacity. These fibers can develop and maintain force higher than type I fibers but less than IIx fibers. Type IIx fibers are called fast glycolytic fibers. They have the highest peak twitch and glycolytic potential yet the lowest oxidative capacity [80]. Rodents and other mammals also express a IIb isoform, which is faster and more glycolytic than the other fiber classes [81]. Typically, human muscles have a set proportion of fiber types per muscle and generally show a mixed fiber population, whereas rodents have a generally fast-fiber composition in most major muscle

groups[82]. In humans motor neurons from fast and slow motor units are dispersed resulting in a heterogeneous muscle fiber type population. The majority of fibers in healthy young adults express a single MHC isoform per fiber. However, as repeated denervation and reinnervation events accumulate with age, the mosaic pure-fiber profile can increase the likelihood of transitions to dual expressing fiber types within a single fiber, leading to a 'co-expressing fiber' [57]. The increase in co-expressing fibers is also seen along side small angular fibers, which are a well-known consequence of denervation[83]. Surgical denervation has been shown to increase the proportion of co-expressing fibers[84], 70% of which have been shown to be positive for Nav1.5, a marker of denervation[17].

While fiber composition is pre-patterned during development and can change with innervation status, activity status also determines the fiber MHC composition. For instance, ~74 year old sedentary individuals have been shown to co-express type IIa/x fibers, which switch to pure type IIa fibers upon exercise training[57, 85]. Interestingly, young sedentary adults have also been shown to have a considerable amount of co-expressing fibers which decrease in frequency with exercise training[86], indicating the particular importance of activity status on fiber MHC expression.

2.2.3.1 Fiber Co-expression and Fiber-type Switching

As previously mentioned, a consequence and indicator of fiber denervation and reinnervation cycles is fiber type co-expression wherein a single fiber exhibits mixed expression of MHC isoforms [57, 87]. Normally, pure fibers express the same MHC isoform per contractile head along the length of a fiber. It has been shown that aged muscle has a large increase in the proportion of co-expressing fibers at ages where

denervation becomes very severe [57]. Additionally, surgical denervation has been shown to increase the proportion of MHC co-expressing fibers [84]. In very old rat muscle 70% of those co-expressing fibers have been shown to be positive for Nav $_{1.5}$ (a marker of denervation) and indicates that denervation is a key contributor to fiber type switching seen in very old age [17]. Adult muscles normally express the Nav $_{1.4}$ sodium channel isoform. Nav $_{1.5}$ is only expressed during development or after denervation and as such is commonly used as a marker for denervation [17]. Beginning from a pure fiber, fiber switch transitions normally go from type I > typeI/ IIa >type IIa>typeIIa/IIx > typeIIx> type IIx/IIb>type IIb (without the IIb in humans)[73]. Co-expression may be conceptually thought of as an incomplete transition during a fiber type switch event, which may arise after a fiber has been denervated and then been reinnervated by a motor neuron from a different fiber class. In addition to the MHC associated changes in contraction, muscle fiber switch has metabolic ramifications as well, which includes a change in the atrophy potential based on the fiber type.

2.2.3.2 Specific Fiber-type Loss

Muscle fiber type can change in response to a variety of stimuli including exercise, stress, trauma and disease. A changing muscle environment in age has been shown to affect type I and II fibers differently in rodents[88]. There are a number of molecular differences in the fiber types, which may help explain this phenomenon but are an area of active research. The most prevailing theory in the literature is that type I fibers are more susceptible to inactivity and denervation-induced atrophy [89, 90], while type II fibers are affected in disease states and aging [88, 91], yet these claims are still in contention. Many attribute the specific responses to disease and aging to the unique

signaling pathways in each fiber type. The most common reason cited for the type I protection from atrophy is due to the actions of the master mitochondrial biogenesis regulator, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC 1α). PGC1α is a factor required for oxidative metabolism, type I biogenesis and mitochondrial proliferation [92]. Interestingly, we have found that type IIa fibers have the highest PGC-1α content[93] yet have the greatest atrophy potential in aging[74], obfuscating the role of PGC-1α in age and fiber-type specific protection in atrophy. It is thought that transforming growth factor beta (TGFβ) family, FoxO family, autophagy inhibition and the nuclear factor kappaB (NF-ϰB) are major drivers in age-related atrophy [91]. NF- ϰB promotes proteasome-mediated degradation through MuRF1 and has been shown to be upregulated in aging and disease [94]. The FoxO family transcription factors upregulate the expression of the atrophy pathway ubiquitin ligases, atrogin-1 and MuRF-1. FoxO is normally suppressed by PGC1α but after disuse or denervation the FoxO induced atrophy pathways supersede muscle-protective PGC -1α [95]. Type IIx fibers are particularly affected in a fast to slow fiber transition and have traditionally been thought to have greater susceptibility to motor unit loss and atrophy [96]. This fact may contribute to the idea that the type II atrophy potential in general is greater than that of type I fibers. Interestingly, overexpression of PGC-1α in dystrophic mdx mouse models both rescued atrophy and induced a fast to slow fiber shift, suggesting that inducing fast to slow shift may be beneficial in at least one neuromuscular disease model [97]. Additionally, with age it seems there is a fiber type specific atrophy that contributes to a reduction in muscle size. There is a 10%-40% loss in type II fiber cross sectional area in the elderly compared to young men [98] with some studies claiming this to be responsible

for the majority of mass loss in age [98, 99]. While some maintain that Type I fibers seem to be largely unaffected in aged humans [100-102], other researchers have shown that the type I affect has traditionally been underestimated while type II size and number loss have been overestimated. Characterization of F344BN rats reveals that F344BN soleus and diaphragm msucles at 3,9, 28 and 30 months do not trend preferentially toward type II fiber loss while they age [103] (further characterized in section 3.2.1). The F344BN rat is a popular aging model and has been validated in that it has a similar aging trajectory to that of humans and longer life expectancies than other models [104]. Furthermore, a study by our lab suggests that the fiber type shift seen in age can be masked by the increase in co-expressing fibers[73]. We argue that the decrease in original pool of type I fibers is underestimated in the literature due to a misclassification of a mixed fast/slow fiber as being a fast fiber, subsequently attributing the status of preferential atrophy potential to a mixed fiber which has a pure slow fiber origin [73]. An extensive study comparing soleus and gastrocnemius muscles in a well regarded rat model of aging concluded that at very advanced age, age related changes in tetanic tension per gram, time-to- half relaxation and shift in MHC expression were of greater magnitude in fast twitch than slow twitch muscles[87]. Additionally, some research shows that the motor unit remodeling in age results in a type II denervation and an increased reinnervation of type I muscle fibers [105-107]. However, this point is also in contention, as a type I to type II fiber type switch has been shown to occur in primarily slow twitch soleus muscle in even in the principally fast twitch rodent models [108].

2.2.3.3 Fiber-type Grouping

In addition to fiber type switching, another phenomenon of muscle aging consistent with high levels of denervation is muscle fiber-type grouping. Fiber type grouping increases with age [109, 110]. Motor neurons determine fiber type during development and fibers of different types are heterogeneously dispersed amongst each other in healthy young muscle (look for reference). Fiber type identity is originally determined during embryonic development by myogenic genes reflecting intrinsic myoblast lineage diversity[111]. This changes as the organism matures and receives neural [112, 113] and hormonal input[114]. Motor neurons can determine fiber type by changing the activity pattern of the muscle contraction, which in turn changes the expression profile of the fiber, resulting in different MyHC and therefore different fiber type classes [114, 115]. Repeated denervation and reinnervation cycles cause fiber clustering due to robust reinnervation from a few motor units [70]. In an early study, quantitative assessment of fiber type distribution in men in three age groups revealed that fiber type grouping could be considered a good proxy measure for repeated cycles for innervation and denervation [116]. Other studies confirmed this [17] while some suggested that an increased rate of grouping after the age of 60 also coincided with the accelerated loss of motor neurons from the spinal cord [109]. This would imply that grouping results from collateral reinnervation as an adaptive response to motor unit loss. Interestingly, grouping occurs in various models with compromised neuromuscular junction stability where the axon and motor neuron are unperturbed [39, 41, 117], alternatively suggesting a myocyte-driven mechanism for collateral reinnervation leading to fiber type grouping.

2.2.3.4 Grouped Fiber Atrophy and Fiber Angularity

A major consequence of fiber type grouping is grouped fiber atrophy. Grouped atrophy steeply precipitates muscle loss at the most clinically relevant ages when falls and mobility impairment increase [27]. Grouped fiber atrophy in age-related muscle atrophy occurs when denervation reinnervation cycles have caused the same fiber types to group together via adjacent axonal reinnervation. If the axon of the motor unit degenerates and the entire motor neuron dies its associated fibers atrophy. This accelerates muscle loss greatly in very advanced age [118]. Histologically, atrophied fibers can be seen in cross section as losing their characteristic ovoid or round shape and increasing in angularity. The prevalence of accumulated small and angular fibers increases at very advanced age in both humans [78, 79] and animal models [17, 27, 74]. Some fibers fail to reinnervate, resulting in an angular fiber cross section if denervation persists [119]. Between the ages of 40 to 75 angular fibers are dispersed among morphologically normal fibers [83], suggesting that the majority of background denervation before very old age is fiber-specific as opposed to grouped. Grouped atrophy coincides with age-related loss of motor neuron cell bodies in the spinal cord [17, 29, 120, 121]. At the same time, the number of motor units has been shown to decline [71]. While there is a possibility that the reduction in fiber number may suggest a retrograde fiber–induced neuron loss engendered by trophic signal loss, an absence of neuron death in diseases like DMD [122] where fiber death is occurring suggests that this does not occur in all cases. Interestingly, NMJ instability in neurotrypsin overexpressing mice has been shown to be sufficient to induce fiber type grouping and reduce fiber number [39] as well as in cases where sporadic denervation occurs due to NMJ instability as is seen in

ALS [119]. Additionally, changes in the neuromuscular junction have been observed to occur before motor neuron loss in geriatric mouse models [3] and even before myofiber atrophy in rat models [18], suggesting that there may indeed be a limited retrograde fiber-induced innervation restructuring occurring in age. In the sections that follow, an overview of the NMJ structure and its association with denervation will allow for better contextualization of NMJ deterioration in relation to the other mechanisms at play during age-related muscle atrophy.

2.3 The NMJ is Disrupted in Age and Neuromuscular Disease

The neuromuscular junction is another known target of denervation-reinnervation cycles in humans and in animal models [18, 123]. Poor environments brought on by disease and aging result in structural changes of the endplate. In the sections that follow the relationship between denervation and age-related changes in the NMJ will be explored, beginning with the development of the NMJ the study of which first delineated the importance of its structural components.

2.3.1 The Development of the NMJ

Guidance of the axon toward muscle fibers and precise nerve-muscle contact is facilitated through molecular signaling. Axon pathfinding is not controlled by the target muscle but by a series of molecular cues along the path of the motor neuron, which depend on neuronal activity[124]. After reaching the muscle target, the pre and postsynaptic elements continue maturation. The presynaptic end plate expresses a high concentration of proteins involved in calcium-dependent synaptic-vesicle exocytosis and recycling[124]. The post-synaptic space expresses receptors at the crests of the post-

synaptic folds[124]. The synaptic elements, which include the presynaptic nerve terminal, the post synaptic muscle fiber and the perisynaptic glia collectively coordinate the maturation of the NMJ[125]. By the time motor axons reach their targets, muscles show a degree of pre-patterned AChRs on their surface[126-128] indicating that these AChR clusters did not form in response to synaptic activity. It is still being debated whether nerve independent clustering/pre-patterning is responsible for mature NMJ clusters on muscle fibers[126, 127, 129-131]. There is no preferential NMJ formation at pre-patterned AChR clusters on cell culture myotubes[132, 133], however there is some evidence that pre-patterned AChR clusters define the NMJ location in some vertebrate and mammal studies[130, 131, 134, 135]. Post-synaptic maturation is regulated by a number of key molecular mechanisms. One key organizing molecule, the proteoglycan agrin, is required to centralize AChR clusters and exclude them from perisynaptic sites and further maintain clustering after muscle innervation[136]. However, in $Agrn^{-/-}$ mice the pre-patterned AChR clusters that form before innervation are not affected by the lack of agrin and remain clustered[126, 127, 136]. Agrin injection into denervated msucles induces AChR clustering, even if there was a previous site of innervation on the same fiber in another area[137, 138]. At the postsynaptic end plate, agrin triggers MuSK autophosphorylation and plays a critical role in AChR clustering[139]. Strangely, $Musk^{-/-}$ mice have neither AChR pre-patterns nor nerve-induced AChR clustering[139]. Agrin interacts via LRP4 a transmembrane protein and a co-receptor of MuSK to continue clustering AChRs at the NMJ[34, 140, 141]. MuSK induces multiple downstream pathways, inclusing the accumulation of Rapsyn and neuregulin receptors[142]. Rapsyn can also cluster AChRs, dystroglycans and other NMJ proteins and rapsyn null mice have

impaired neuromuscular transmission[143].

2.3.2 Effects of Repeated Denervation on NMJ Structure

The NMJ is composed of a presynaptic motor nerve terminal, an intrasynaptic element (synaptic basal lamina) and a postsynaptic element (muscle membrane). [144]. The basal lamina extends from the synaptic cleft to cover the rest of the muscle membrane. The peri-synaptic Schwann cells insulate the pre-and post-synaptic space, while AChR clusters reside in the post-synaptic membrane. The stability of the AChR cluster can range from very stable to somewhat unstable, indicating the NMJ can respond plastically to a variety of stimuli which helps maintain plasticity of the NMJ in response to nerve damage or exercise [145]. The NMJ structure is normally stable in adult rat muscle with a turnover of 10 to 12 days[146]. AChR turnover is affected by innervation status and neural factors like agrin. After denervation, the half-life of the original AChRs drops to 3-7 days while new AChRs inserted into the membrane have a half life of 1 day (mouse)[147] or 2-4 days (rat)[148, 149], matching the half-life of extrajunctional AChRs. After reinnervation and also with normal electrical stimulation, expression of unstable AChRs is blocked and expression of new stable AChRs is increased[148, 150-152]. However unlike normal electrical stimulation, reinnervation can uniquely restore the half life of already-present AChRs back to pre-denervation levels, indicating that there is a neural trophic factor necessary in addition to electrical stimulation which increases the stability of AChRs already embedded in the post synaptic membrane[149]. Interestingly, the stability of AChRs in muscle fibers transfected with neural agrin cDNA is increased in a dose-dependent fashion, raging from 1 to 10 days [153].. Simultaneously, NMJ fragmentation [154, 155] and gutter depth observed through a

TEM[154] increase after denervation in rats injected with Botulinum toxin A. In direct NMJ ablation via fiber damage or methyl-bupivicane injection, AChR clusters begin to disappear immediately[61, 156, 157], presumably due to phagocytosis. 6-12 days after laser ablation, no AChR-αbungaro-toxin (αBTX) signal could be picked up by a CCD[158]. Interestingly, enhanced photos of ablated fibers show that there are AChRs, which persist at the endplate even 11 weeks after ablation with an altered morphology[61] indicating that incomplete loss of the endplate after serious fiber damage may allow for reinnervation well after initial trauma. Interestingly, Li and Thompson suggested that nerves and glial cells remain in direct contact with the basal lamina up to 3 days post-ablation, indicating no immediate retrograde nerve damage as well as persistent attachment to the old synaptic site despite NMJ damage[61]. Pre-synaptic changes like axonal disappearance are more evident than post-synaptic changes [159]. This can be observed at the EM level in the event of a nerve pullback as one would see in an ALS model, where a denervated NMJ completely lacks a motor neuron 'terminal bouton' over the postsynaptic secondary folds where the AChRs reside [25]. Beginning 2-3 days post ablation, labelling with a different color AChR- αBTX shows that as the fiber regenerates, new pockets of AChR begin to be deposited near the old AChR sites. The number of new sites peaks at 6 days post-ablation. The authors reasoned that the punctate AChR labelling commonly cited in the literature was due to a regenerating myofiber[61]. There are a number of changes that occur to the NMJ 2-3 days post fiber damage as the fiber begins to regenerate. First, some parts of the fiber that were previously synaptic and contained AChR clusters will become covered with a regenerating synaptic basal lamina. Second, the nerve terminal sends out extensions to connect with the newly formed AChR

puncta. Schwann cell extensions were associated with these new terminal growths. Generally, from day 12 to 6 weeks out, the AChR fragmentation patterns increase as the junction expands in size. Li and Thompson also noticed a variety of changes, which occur when a motor neuron reinnervates a fiber. From day 20 to day 50 post-ablation, the peripheral Schwann cells (pSC) and Nerve Terminal (NT) lose secondary branches and the AChR sites beneath the lost NT branches dissipate. From day 50 to day 80, simultaneous pruning and branching of secondary branches occurs for both the NT and pSCs. New receptor sites appear beneath newly branched NT and pSC regions. At this point, AChRs appear fragmented and the nerve terminals become varicose[160]. Unfortunately, to our knowledge there are no other studies examining the temporal changes of the NMJ after a pre-synaptic event in the amount of detail performed by Li and Thompson noted above. One study examining some of the changes in endplates in a spinal muscular atrophy (SMA) model examined pSC and motor neuron changes in an SMA model over time[161]. The issue with using this SMA model is that it affects the initial development of the endplate and therefore makes it more difficult to compare to changes in a model where damage to the NMJ is induced during adulthood. One proposed area of research would be to compare the temporal changes of the endplate that are a result of myocyte-induced damage to those due to nerve-induced damage. In turn comparing the damage due to these conditions to the damage seen in age over time would be a suitable way to monitor whether damage in age more closely resembles damage due to fiber damage or motor neuron damage. This could be achieved comparing the changes in different animal models. For instance the Robataille lab has expertise in motor neuron transfections and could induce motor neuron defect in various models, like the Cas-BR-E

murine leukemia virus (MuLV), which induces degenerative myeloencephalopathy. Another suitable option could be to use a nerve crush model. Comparing such a model to a fiber damage model as used Li and Thompson, and then comparing these to wild type models could allow for a better understanding of the source of NMJ fragmentation in age.

Post denervation events can be divided into early and late stage changes. Early stage events create a microenvironment that is conducive to reinnervation [159]. These acute changes include up-regulated expression of myogenic regulatory factors (MRFs), AChRs and Schwann cell proliferation [154, 159, 162-166]. Given ample time to reinnervate, the *tibialis anterior*(TA) of *thy1-YFP16* mice after a sciatic nerve crush first exhibit motor nerve recovery 2 weeks post crush, and from 3-6 weeks undergo a period hyper reinnervation wherein there is a >1 axon:endplate branching ratio. These *thy1-YFP16* mice transgenic mice express high levels of yellow fluorescent protein in motor and sensory neurons and can be used to examine adult motor neurons. After 3-6 weeks, the axon:endplate settles to 1:1 and the terminals resemble those from uninjured mice [167]. Should denervation persist, a number of late stage changes make it more difficult for reinnervation to occur: Schwann cell numbers decrease, muscles atrophy [159] and an infiltration of connective tissue between muscle fibers prevents regenerating axons from entering into intramuscular nerve sheaths [162]. At the NMJ, fragment number and gutter depth increase, prompting a reduced efficiency of synaptic transmission [154]. A simultaneous decrease in muscle fiber conduction velocity (MFCV) [168] decreases the efficiency of the synapse. Finally, the mRNA expression of AChR and MRFs is initially up-regulated before normalizing to background levels [154, 163, 169-171].

Considering the evidence that postsynaptic NMJ structure responds to denervation, it is likely that there is a direct 'denervation signal' processed by the NMJ, which results in NMJ remodelling. Moreover, since denervation is in many cases responsible for fiber atrophy, identifying the mechanisms of NMJ structural adaptation are key to determining which molecular rearrangements ultimately lead to fiber atrophy. While there are many structural molecules constituting the NMJ the underlying structure of the sarcolemma is folded through actin remodelling [172, 173]. As such, researchers have determined that one point of convergence between the presynaptic and postsynaptic space is a proteoglycan called z-agrin, which remodels actin through rapsyn[174] and modulates the elastic response of the NMJ through the MuSK/agrin/rapsyn signalling axis [35].

2.3.3 The Agrin-MuSK Axis

Neural agrin from the motor neuron maintains the integrity of the NMJ by phosphorylating and activating MuSK through its co-receptor LRP4 [35, 38, 175, 176]. AAgrin is most notably known for its AChR clustering properties in development, as elaborated on is section 2.3.1. In the absence of neural agrin, the NMJ disassembles within days [37, 38]. Agrin is critical for NMJ stability and also initiates AChR clustering during development [144]. Activated MuSK then interacts with Dok7, in turn enabling MuSK phosphorylation and further activation, a sequence required for AChR clustering during NMJ maintenance and during development [177]. Other effectors downstream from MuSK become activated, including tyrosine kinase Abi1 and metalloenzyme geranylgeranyl transferase I (GGT). GGT facilitates Rho GTPase activation and in conjunction with other pathways, regulates actin dynamics required for AChR trafficking

and membrane insertion [35, 178]. Beyond MuSK mediated AChR density control, Agrin acts to stabilize the AChR lipid domains through an effector called rapsyn. Agrin interacts with rapsyn through Adenomatous Polyposis coli (APC), effectively slowing the highly unstable rapsyn kinetics and allowing anchoring of the AChR to the membrane in lipid AChR rafts [35, 179]. There are a number of agrin signalling regulators including MuSK endocytosis[180], lipid microdomains [33] and intracellular calcium [181]. Since the agrin-MuSK pathway is key in maintenance of the NMJ and its response to denervation, studies have recently explored whether this pathway is changed in age-related muscle atrophy and in other neuromuscular diseases [2, 31, 39].

2.3.4 Disruption of the Agrin-MuSK Pathway Occurs in Aging Humans and in Disease Models

Determining the proximate causes and the primary effects of NMJ disruption are an intrinsic difficulty in studying a structure that interfaces with and is regulated by an input (neuron), output (myocyte) and medium (synapse). Despite the signalling convergence, the saying that 'structure begets function' is particularly important when studying the NMJ. In line with this and in addition to the changes presented above, a recent survey of the genome and proteome of aging rats identified the structural components of the NMJ, like AChR, MuSK and Lrp4 are significantly upregulated in age [31]. Previous studies showed that synaptic genes are known targets of denervation [182, 183], indicating that changes in NMJ genes are a biomarker of denervation. Additionally, these studies implicate the agrin-MuSK axis of NMJ instability in age-related muscle atrophy. Models of NMJ instability have substantiated this claim. After 2-3 months of tamoxifen-induced agrin depletion, conditional agrin knockout mice showed marked

NMJ deterioration [184]. Additionally, a clinical trial indicated that 38% of those with age related muscle atrophy had elevated cleaved agrin fragments in their serum[185]. While these studies indicate the promise of agrin therapy in treating age related muscle atrophy, there are clearly other factors contributing to NMJ disruption and age related muscle atrophy [185]. Some of these other mechanisms have been explored through the use of various animal models. This is clearly exemplified in a neuromuscular disease called myasthenia gravis (MG). Patients suffering from MG create antibodies to either MuSK, AChR, Dok7, rapsyn, laminin and AChRε, with the resulting phenotypes recapitulating many of the NMJ and musculature defects seen in age-related muscle atrophy. The more common anti-MuSK MG directly results in NMJ fragmentation [186-188]. ALS and severe age-related muscle atrophy have many phenotypic similarities [189]. Patients with ALS have severe muscle weakness and atrophy, eventually losing diaphragm control, which results in death [25]. Although it is largely considered a motor neuron disease, NMJ maintenance of the $Sod1^{G93A}$ ALS model via MuSK overexpression has been shown to partially rescue ALS paralysis and reduce symptoms [190]. Dystrophy also presents an opportunity for comparison. A comprehensive review by Rudolf [2] delineates the many similarities between dystrophic and aged neuromuscular systems, particularly at the NMJ. While direct involvement of aberrant agrin-MuSK signalling has not been extensively studied in the context of dystrophy, a decrease of downstream effectors of the pathway like cAMP/adenylate cyclase 2, PKA type I α, and phosphodiesterase 4a were shown in a proteomic analysis to be correlated between age/dystrophy and muscle loss [31]. Finally, a disease called slow-channel syndrome (SCS), which directly affects the acetylcholine receptors, culminates in an electrical

profile similar to that of a long-denervated and recently reinnervated NMJ. SCS AChRs have mutations forcing a slower Ca^{2+} gating resulting in a longer cation dwell time. As explained in the 'mitochondria –calcium dysregulation' section below, this leads to organelle and transcriptional instability, as well as a decrease in synaptic folding [191]. Interestingly, AChRγ, which increases following denervation, has been shown to have a slower conduction time and higher turnover [192] which increases cation load. Furthemore, mice shown to be lacking AChR epsilon subunits in favour of gamma subunits die soon after birth [193]. Interestingly not only is the foetal isoform AChRγ upregulated after a denervation event [170], we have shown this isoform to be upregulated in aging rat muscle as well[17]. These data suggest that nonspecific upregulation of the NMJ components may be a response to denervation or an attempt to compensate for decreased neural input and implicate Ca^{2+} in fiber dysfunction brought on by denervation-initiated NMJ destruction. To further understand the causal relationship between molecular mechanisms and the ensuing deterioration of the NMJ the next sections will explore the mechanisms that some have proposed lead to NMJ instability in age-related muscle atrophy.

2.4 Mechanisms Leading to NMJ Defects in Age

It is unclear what causes NMJ fragmentation with aging. Some have proposed that degeneration, occurring first at the fiber underlying the synapse, and its subsequent regeneration could fragment the myofiber NMJ and then the presynaptic NMJ through retrograde signalling (of MuSK co-receptor Lrp4 [140]) and structural adaptation. This has been proposed in dystrophy [194] as well as in aging models [61]. Another possible

cause of fragmentation is motor-neuron death or damage, which accelerates after the age of 60 [58, 120, 121]. Simultaneous death of myofibers has also been observed to occur at a similar rate and time point [195], suggesting a motor neuron driven mechanism for age-related muscle atrophy. This is consistent with the observation that NMJ fragmentation occurs suddenly after traumatic denervation events [61]. Yet, rodent models have shown that the loss of pre and postsynaptic structures can occur over time, independent of motor neuron loss [3], and even before a denervation event occurs [18, 30]. While the sources of NMJ disruption have been shown to occur from both the myocyte side and neuron side of the NMJ (explored previously in 'grouped fiber atrophy and fiber angularity'), simultaneous input from many sources is likely occurring in age. Likely simultaneous pre- and post -synaptic mechanisms include organelle disruption and second messenger signalling like Ca^{2+} (which will be explored in the last section). One of these inputs occurs through neurotrophins, which have been proposed as a neural mechanism of NMJ structural remodelling.

2.4.1 Neurotrophic Factors

Neurotrophic factors play a role in NMJ development, maintenance and plasticity. Neurotrophins also modulate synaptic transmission and activity [196, 197]. The role of trophic factors and how their activity changes or responds in age are actively being researched. Among the growth factors that play a role in NMJ plasticity are brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), cytokines like glial-derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CNTF), and other factors like insulin-like growth factor (IGF-1 and IGF-II) and fibroblast growth factor (FGF) [62]. The functions of these factors and their receptors on the motor

neurons are modulated in age and denervation to varying degrees. The expression of TrkB and TrkC (BNDF, NT-3 and NT-4 receptors) have been shown to be down regulated in age, while the GDNF receptor components GFRα1 and Ret have been shown to be upregulated[198]. These regulation patterns match the neurotrophin levels in ageing motor neuron environments. For instance, GDNF levels have been shown to increase in aging mouse motor neurons, likely as a compensation for receptor targeting failure[199]. Regarding denervation, GDNF supplementation has been shown to help prevent motor neuron loss after injury 1995[200], and denervation causes an up regulation of GDNF in rat and human muscles[201]. Neurotrophins are also important for NMJ maintenance. BDNF, NT-3, and NT-4 are important for the maintenance of AChR clustering at the NMJ [42, 202, 203]. If neurotrophin expression or signalling is disrupted, AChRs can be dispersed resulting in NMJ fragmentation and increased muscle fatigability [42]. Interestingly, groups have shown that IGF-1 can attenuate age-related sporadic disruption of NMJ morphology compared to controls [204, 205] although the ages of the 'old' mice in these studies (20-24 months) was not old enough for persistent denervation to be a significant contributor to muscle loss at this point. Synaptic factors are also known to organize postsynaptic endplate structure via motor neuron release of epidermal growth factor (EGF) and neuregulin. EGF and neuregulin enable clustering of the post-synaptic AChR while promoting transcription of NMJ structural genes [206]. Neuregulin has also been shown to be an exercise-induced myokine that promotes myogenesis and regulates muscle metabolism [207]. Because exercise and caloric restriction can slow the aging process by recuperating an age-related decline in autophagy[60], neuregulin upregulation may be a target for treating age related muscle

atrophy as well [208, 209]. Growth factors acting across a synapse act as a distal modifier on post-synaptic organization. In addition to the neural sources (agrin and the neurotrophins) of post-synaptic structure control, there are a number of myocyte control mechanisms that act in concert as more proximal regulators of NMJ structure.

2.4.2 Mitochondrial Dysfunction and Oxidative Stress in Age Related Muscle Atrophy

2.4.2.1 Mitochondrial Oxidative Stress

The oxidative stress theory of aging states that there is a gradual decrease in cellular function due to accumulation of oxidatively damaged molecules. The damaged molecules reduce protein function and compromise the DNA replication process [210]. Mitochondria create reactive oxygen species as a by-product of oxidative phosphorylation. Oxidized guanosine levels, a marker of oxidized DNA, are higher in mtDNA compared to nuclear DNA. This is due to the closer proximity of mtDNA to the reactive oxygen species (ROS) source and the lack of mtDNA excision mechanisms [211, 212]. Mutation of critical electron transport genes decrease the efficiency of electron transfer, resulting in higher ROS generation, thereby perpetuating the ROS cycle[210, 213]. Increased oxidation has also been shown to occur in aging muscle[214], yet the question remains whether oxidative damage contributes to NMJ dysfunction or if the oxidative damage is itself secondary to NMJ deterioration. Many groups have studied the relationship between age related muscle atrophy and oxidative damage [215-217]. The interaction between chronic inflammation, oxidative stress, and mitochondrial dysfunction have been proposed to collectively result in muscle atrophy[218]. Under

normal conditions, muscle mass is maintained via an equilibrium between protein synthesis and degradation. Oxidative damage is thought to disrupt protein synthesis and increase breakdown [219]. Recent evidence has linked the increase in ROS to disruption in the anabolic IGF-1/PI3K/Akt pathway[208, 220] and aging rats possess reduced sensitivity to a downstream effector of Akt called mammalian target of rapamycin (mTOR), a critical regulator of muscle cell size and protein synthesis[221]. The transcriptional coactivator PGC-1α is another element critical to anti-catabolism, which regulates mitochondrial content, respiration, and inflammation [222]. PGC-1 α is thought to prevent catabolism in aging muscle via FOXO3 inhibition [95] and NMJ maintenance [223]. In addition to improving mitochondrial respiration, PGC-1α is thought to reduce ROS damage via antioxidant and uncoupling protein upregulation[224]. In order to determine the effects of ROS on the NMJ, Jang et al used *sod1* deficient mice (Cu/Zn superoxide dismutase; CuZnSOD) and found many similarities between aging and sod1 deficient muscle [215]. *Sod1*$^{-/-}$ KO mice exhibit similar NMJ disruption and muscle phenotype to aging mice but at an earlier time point. *Sod1*$^{-/-}$ mice NMJs show fragmentation and similar morphology at 11 months when compared to 33-month-old wild type mice. Motor neurons also exhibit extensive sprouting and thinning of axon terminals in *Sod1*$^{-/-}$ mice, similar to what is seen in aging muscle [215, 217]. These data suggest that oxidative stress may contribute to NMJ decline in age. *Sod1* $^{-/-}$ mice also exhibit a shift from fast to slow fiber type and a grouping of slow fibers [225]. What may be even more telling are the mitochondrial changes observed in the *Sod1* $^{-/-}$ skeletal muscle. Mitochondrial ROS in *Sod1*$^{-/-}$ mice is significantly increased compared to wild-type mitochondrial ROS emission. Increased ROS has an effect at the NMJ itself.

Presynaptic H_2O_2 is stable and membrane permeable impairing presynaptic release of acetylcholine through the action of the vesicle fusion protein SNAP25 [226]. Although mitochondrial ROS may also be elevated in aging muscle [227], denervation itself can cause an increased mitochondrial ROS release [40] and thus the extent to which an increase in mitochondrial ROS with aging may be a cause versus an effect of denervation is unclear.

The NMJ is particularly susceptible to mitochondrial damage as there are high populations of mitochondria at the presynaptic terminal, as well as at the subsarcolemmal region beneath the NMJ [228]. The mitochondria in these regions serve multiple functions, including energy generation, calcium regulation, synaptic transmission, and apoptosis. For instance, disrupting energy maintenance via muscle-specific upregulation of uncoupling proteins (UCP1) significantly disrupts NMJ morphology and is sufficient to cause motor neuron degeneration [229]. In the presynaptic space, mitochondria are shuttled along microtubules by kinesin and dynein motors[230]. Complex filamentous linkages stabilize mitochondria-vesicle interactions and further organize mitochondria, facilitating ATP generating and Ca^{2+} buffering function[230, 231]. Defects in either transport or mitochondrial stabilization machinery, as seen in SOD1 and TDP43 ALS models, result in severe NMJ defects and tell-tale signs of mitochondrial abnormality, such as swelling and fragmentation [214, 232, 233]. Mitochondrial disruption is seen in the ALS model $SOD1^{G93A}$, where isolated subsarcolemmal mitochondria have a decreased ATP generation capacity, increased oxygen consumption and increased ROS generation compared to their wild-type counterparts [234]. Whether mitochondrial changes in aging muscle are a primary change due to organelle dysfunction versus a normal physiological

response to denervation in aging muscle remains a point of controversy. In support of the latter idea, mitochondrial dysregulation and time to pore opening are altered in aging muscle [235] and this is similar to the response to denervation which also sensitizes the mitochondria to transition pore opening [236]. Mitochondria had been implicated in skeletal muscle atrophy and dysfunction with aging. A previous study in our lab confirmed that calcium retention capacity decreased by 50% with aging indicating that there was an increased sensitization of the mitochondrial permeability transition pore (mPTP) to apoptosis[235]. This mitochondrial pore forms under conditions of extreme stress (high calcium load) and indicates that the cell is undergoing apoptosis. The study also indicated that older muscles had an increased likelihood of having high endonuclease-G, a marker of mitochondria pro-apoptotic factor[235]. Furthermore, a repeated nerve crush study used TEM to analyze the NMJs beneath nerve-crushed *intraplantar* muscle. They noticed an accumulation of subsarcolemmal mitochondria at the post-synaptic site. Additionally, there were an increased number of large mitochondria with disorganized cristae[237], which the authors compared to findings in myasthenia gravis[53, 238]. The authors suggested that the characteristics could be a reflection or a consequence of impaired synaptic transmission. In contrast, it has also been suggested that calcium buffering and oxidation of the NMJ contributes to NMJ remodelling and decline in innervation. In support of this point, Zhou et al demonstrated that mitochondria adjacent to the AChR are selectively depolarized when challenged by calcium in a mouse model of ALS, which exhibits significant neuromuscular degeneration [239]. In the following section, the critical calcium-buffering role of

mitochondria will be explored as a possible medium linking denervation and junction disruption.

2.4.2.2 Mitochondrial Calcium Dysregulation

Mitochondria play a role in buffering cytosolic Ca2+ released by the sarcoplasmic reticulum. Evidence that cytosolic Ca^{2+} increases with age suggests that ER-mediated apoptotic pathway should stress and induce myocyte apoptosis, yet it is unclear if this is the cause of myocyte apoptosis in age [240]. More likely signs point to caspase-2 and c-Jun NH(2)-terminal kinase (JNK) mediated apoptosis, which are activated by calcium and oxidative stress[241, 242]. The links between calcium dysregulation and aging are unclear, yet much can be learned from neuromuscular disease models with similar defects to age. Calcium dysregulation and neuromuscular disease is extensively studied through dystrophinopathies, like DMD. Dystrophies are the result of a contraction-induced rupture of the sarcolemma, causing Ca^{2+} imbalances and widespread organelle damage [243]. Excess calcium caused by ruptures and leaky Ca^{2+} channels [244, 245] can lead to protease activation [246] and oxidation through cytosolic and mitochondria sources [247-249]. Increased Ca^{2+} is buffered by the sarcoplasmic reticulum, and then the mitochondria, which has been shown to result in damage at these sites [250]. These mitochondrial effects are also seen to result in faulty energy maintenance and metabolism [251]. Calcium dysregulation is of interest because it may directly disrupt the NMJ. For instance, in age some fast fibers become dependent on extracellular Ca^{2+} to maintain tetanic force [252]. Perhaps more importantly, a disruption in excitation-contraction coupling has been shown to occur in aged muscles [105, 253]. A similar effect has been shown to occur at the NMJ of SCS patients brought on by an increased calcium dwell

time caused by a faulty AChR profile [191]. Thus there is evidence that calcium disequilibria may have direct effects on the age-induced dispersal of the NMJ. Furthermore, an increase in cytosolic Ca^{2+} may activate Ca^{2+} dependent proteases such as calpain. Normally, the NMJ structural protein rapsyn interacts with calpain and inhibits its proteolytic functions [254]. However, given some evidence in the literature we suspect that a possible age- dependent alteration of rapsyn signalling and an increased Ca^{2+} dwell time due to compromised mitochondrial buffering[235] may increase protease activation and further disperse the NMJ [254].

2.5 Conclusions

The importance of aging research continues to grow in tandem with the growing aging population's healthcare needs. Given the high incidence of muscle and metabolism-related injuries among the elderly, costs are projected to rise [14]. While the most obvious effects of muscle wasting include a loss of independence, increasing muscle mass would extend beyond restoring every day mobility. Abnormally low muscle mass has also been shown to exacerbate disease, disability and mortality in the elderly [12]. Preventable diseases are also thought to be exacerbated by muscle loss. For instance the loss in glycolytic fibers in age is thought to contribute to middle age diabetes and hepatic steatosis [255]. Selective increase of glycolytic muscle has been proposed as a mechanism of reversing muscle fiber type II atrophy while both increasing metabolism and carbohydrate utilization instead of fat storage [255]. These studies and many others show that commensurate with its widespread involvement in many diseases, the exact mechanisms of age- related muscle loss continue to be of interest to the scientific

community. To navigate complexities of age related muscle atrophy the aging muscle community has placed a high importance in studying the most likely causes of muscle atrophy. The well-established impact of denervation as a cause of muscle atrophy makes it an attractive target for future research and investment [27]. Research has implicated NMJ instability as a probable cause of denervation in age and in turn NMJ instability makes it more difficult to reinnervate and likely precipitates severe fiber atrophy[209] [2]. The Agrin-MuSK pathway has been shown to be particularly important in deciphering how NMJ instability and denervation relate to age related muscle atrophy [31]. While the aetiology of muscle mass loss is complex, disease models continue to inform the community on potential mechanisms and methods of treatment for age related muscle atrophy.

CHAPTER 3: EXPERIMENTAL ARTICLE

3.1 Introduction

3.1.1 Introduction

Increased frailty[10], impaired metabolic response[255] and an increase in disability [8, 96]are common results of a decrease in muscle mass with age. Severe muscle loss at very advanced ages (≥80 y) results in a loss of independence and an increase in co-morbidities in a variety of diseases[47, 102]. As such, studying the mechanisms of age-related atrophy are critical for unburdening our healthcare system[11] and restoring functionality to the very old.

One of the primary culprits of muscle loss in age is denervation. In young age there is background denervation accompanied by reinnervation, but with time, the frequency of sporadic denervation progresses and the likelihood that reinnervation occurs decreases in humans[78] and rats[17]. There are a variety of changes in ageing muscle, which support the idea that there is an increase in the number of denervation-reinnervation cycles in age. An increase in fiber type grouping[109, 110], fiber type switching [57], very small angular fibers[57, 78, 79], and in fragmented NMJs[56, 256, 257] all seem to indicate that failed reinnervation is a main driver of muscle mass and remodelling in age.

There are a variety of proposed mechanisms for denervation. Among them, an increased likelihood of motor neuron death[120] and motor unit loss[70, 209], as well as fiber damage[61] have also been shown to occur with age-related muscle loss. An important observation in ageing muscle as well as in many diseases is that there is

significant fragmentation observed at the NMJ[155] and that some of the proteins related to its structural maintenance could be disrupted in age[31] This has led to the hypothesis that disruption of the pathways maintaining the NMJ, such as the agrin-MuSK signalling axis, may be contributing to sporadic denervation as well as a driver of NMJ fragmentation in age.

In our study we used a neurotrypsin-overexpressing model of sporadic denervation called the Sarco mouse. The over-expression of neurotrypsin increases agrin cleavage, resulting in impaired MuSK activation and increased AChR fragmentation[39]. We used the Fischer 344 Brown x Norway rat (F344BN) as an aging model as this has been shown to exhibit a similar muscle aging trajectory as humans[44]. The Sarco mouse exhibits many similar phenotypes to those seen with normal aging, which has been used to validate the Sarco mouse as a model of sporadic denervation and as a model that closely recapitulates age-related muscle deterioration [39]. Furthermore, 8-month-old Sarco mice have robust reinnervation considering their maintained motor neuron number and fiber type grouping[39] and this likely accounts for the relatively modest muscle atrophy seen in this model. As such, we used the Sarco mice as a standard to calibrate the changes we saw at the aging versus young adult NMJs, noting that any differences or similarities we observed would allow us to explore the involvement of the agrin-MuSK pathway in aging muscle.

3.1.2 Hypothesis

Disruption of the agrin-MuSK pathway is a primary cause of NMJ fragmentation in age.

3.2 Methods

3.2.1 Rationale for rodent models

We used the Fischer 334 x Brown Norway f1-hybrid (F344BN) rat to investigate the effects of age on the neuromuscular junction. These animals show a similar aging trajectory to humans[104]. The 50% mortality at 146 weeks of age is longer than the 130 week 50% mortality of the Brown Norway x Fischer 344 and significantly longer than the 103 week F344 rat strains[44]. F344BN have a number of aged neuromuscular phenotypes present at an age of 36 months, which correlates to a clinically relevant age of 85 years in humans[44]. Physiological indicators of age-associated muscle atrophy in humans and rodents include muscle mass loss[99, 258], fiber type grouping[99], fiber angularity[27] and NMJ fragmentation[32, 39, 60, 184, 259]. The aging trajectory of sarcopenia in F344BN rats has been characterized in various muscles including the *vastus lateralis* (VL*)*, *rectus femoris* and *vastus medialis* [104]. The VL is important for daily function and also exhibits a high degree of fiber type coexpression, fiber diameter loss and general atrophy in elderly humans [57, 107]. The VL muscles are also large and easily isolated in rats. For these reasons we analyzed changes between young adult (YA, 8 month old) and very old (VO 36 month old) rats in the VL muscle.

The Sarco mouse model of premature muscle aging was used to see if the effects of a specific post-synaptic defect resulted in muscle changes which were comparable to those seen in the aging rat models. The muscle defects in the 8-month-old sarcomice are directly a result of neurotrypsin-induced NMJ instability. Inducing NMJ instability in the sarcomouse via neurotrypsin overexpression results in agrin cleavage causing decreased MuSK signalling and increased AChR fragmentation at the NMJ[37, 39, 260]. Both

Sarco mice and very old animal models share many phenotypes, like fiber type grouping, fiber type switching and fiber co-expression[37] but the specific defects at the NMJ have never been studied before in great detail in either model, let alone compared. While we have previously suggested that the muscle fiber characteristics in very old muscle are due to a large amount of denervation-reinnervation [17], the specific post-synaptic defect in Sarco mice seems sufficient to cause some, but not all of the aging characteristics[84]. For instance, while Sarco mice have high proportion of grouped fibers, these fibers are unchanged in size, suggesting that the post-synaptic defect is not affecting the ability of the Sarco mouse to reinnervate at a robust rate. Additionally, the motor neuron count in the Sarco mouse is unaffected[39], further supporting the use of the model as a tool to assess whether NMJ deterioration in age is caused by a specific post-synaptic defect.

3.2.2 Ethics statement

All experimental procedures on animals were made with prior approval from the McGill University Animal Care committee (2012-7189). The University of Calgary Animal Care Committee (BI09R-11) previously approved the procedures on the F344BN rats as well.

3.2.3 Animals

Male F344BN f1 hybrid rats were purchased from the National Institute on Aging NIA facilities at Harlan Indianapolis, IN, at 8 months of age (n=8; 399.86 ± 9.04g (X ± SE)) and at 36 months of age (n= 9; 581.14 ± 46.25g). Animals were housed in a 12:12 light dark cycle for at least 48 hours following arrival. Animals were anesthetised with 55-65mg/Kg of sodium pentobarbital before muscle harvesting. To study the impact of

sporadic denervation we used Sarco mice (23.92 ± 0.82g; n=5) and wild type mice (43.35 ± 2.86g; n=3). Sarcomice (Neurotrypsin over-expressing C57BL/6 mice) were provided by Neurotune, Switzerland, and bred at the Research Institute of the McGill University Health Centre vivarium. All of the mice were heterozygotes until backcrossed with wild type C57BL/6 mice. In all experiments, 8-month-old mice were used to study the impact of sporadic denervation and reinnervation in young adult muscle, given that the reinnervation response is robust at this age. Animals were sacrificed with CO_2 asphyxiation followed by cervical dislocation.

3.2.4 Surgical Procedures

F344BN rat VL and sarcomouse *soleus* were dissected and removed completely from the animal. The right *soleus* muscle was dissected free of adipose and connective tissue and a portion was mounted in tragacanth gum, before being frozen in liquid nitrogen-precooled isopentane and stored at -80˚C until sectioning. For F344BN rat experiments, each muscle was mounted on cork using OCT mounting medium, frozen in liquid nitrogen-precooled isopentane and stored at -80˚C until sectioning.

3.2.5 Cryostat Sections

Rat and Sarcomouse sections were cut using a Leica CM-3050-S cryostat (-20˚C). 10µm thick muscle cross-sections were mounted onto glass slides, and left to air dry for 2 hours before being stored at -80°C until use.

3.2.6 Immunofluorescent Labelling of the NMJ

Sections were defrosted in slide boxes for 30 minutes and set to air dry for 1 hour. Tissues were fixed in acetone for 15 minutes at 4˚C and then washed (2x5 minutes PBST,

1x PBS) before being incubated in permeabilization solution (0.1% triton in phosphor buffered saline (PBS)) at room temperature for 15 minutes. A second wash was performed and sections were subsequently incubated in blocking solution (1% normal goat serum (NGS) and 5% bovine serum albumin (BSA)) for 30 minutes. Incubations with α bungarotoxin (B13422 Life Technologies; 1:200), anti-MuSK (rabbit polyclonal, Abcam AB5619 1:100), and anti-rapsyn (mouse monoclonal, Abcam Ab11423, Sigma 1:750) were performed at room temperature for 2 hours in a dark humid chamber, with all dilutions being performed in blocking solution. Slides were washed again and the respective secondary antibody was diluted in blocking solution and applied for 1 hour at room temperature (AF350 IgG1 goat anti mouse 1:100, AF594 IgG Goat anti rabbit 1:100). A final wash on the sections preceded mounting with coverslips using prolong gold. Images of the whole muscle cross section were captured on an Axio Imager M2 (Zeiss) and analyzed using imageJ software (U.S. National Institues of Health, Bethesda, MD, USA).

3.2.7 Deconvolution

Images were deconvoluted on Autoquant X3 using the 'blind deconvolution' setting before NMJ intensity and morphometric analyses were performed. This was done by entering image parameters provided by the Axio imager M2 metadata and then by subsequently performing a combined 3-channel deconvolution for other analyses not presented here. A separate, single-channel deconvolution was performed on the same raw images for NMJ intensity and morphometric analysis. Deconvoluted images were exported to Imaris v 8.1.2. Statistics were performed in GRAPHPAD Prism (GraphPad Prism, Inc. La Jolla, CA, USA) across wild type (WT) and Sarcomouse (SARCO) groups

as well as in young adult and very old groups using non-parametric t-tests. Sarcomouse, wild type, young adult and very old groups were coded to blind the data collector. $P<0.05$ was considered statistically significant.

3.2.8 Image J Analysis

Images were opened channel-by-channel. NMJs were traced separately on each channel semi-automatically using a set threshold value per channel. Outlines of each NMJ object were chosen using the wand tool after turning the 16-bit image into a binary image. Outlines of the thresholded objects were used to gather intensity values across a series of WT and sarcomouse images after they were matched to the original 8-bit images. NMJ intensity values and areas were collected for each image on their respective MuSK, AChR and Rapsyn channels. Fibers were also traced using the Wand tool. Fiber outlines were observed visually using the MuSK background staining. Data was compiled in Microscoft Excel. All groups were coded to blind the data collector.

3.3 Results

3.3.1 Animal Characteristics

In this study we used young adult (YA; 8 month old) and very old (VO; 35-36 month old) Fischer 344 Brown-Norway rats obtained from the National Institute on Aging (Bethesda, USA). We also used Sarco mice (SARCO; 8 month old) provided by Neurotune (Schlieren-Zurich Swizerland) and wild type C57BL/6 mice (WT; 8 month).

3.3.2 Muscle Fiber Size

It is well know that muscle fiber mass decreases in age[79]. The mass of the VL decreased by 42.64% from 1723.91 ± 46.58mg (X ± SE) in YA to 988.99 ± 34.31mg; $P<0.05$ in the VO group. The *soleus* muscles weighed 9.83 ± 0.47mg in the WT and decreased by 33.67% to 6.52 ± 0.28mg in the SARCO. Fiber size distribution was quantified in aged rats and Sarco mice (with an average of 582 ± 7.89 fibers counted in in YA, 570.5 ± 6.32 in VO, 278 ± 46.84 in WT, and 178 ± 58.96 in SARCO). A large accumulation of small angular fibers coincides with the acceleration of muscle mass loss in ageing muscle[17]. Furthermore, we have previously shown that >90% of fibers ≤ 1000 μm^2, a size which is scarcely seen in young adult muscle (represents the first percentile of the distribution in young adult muscle), express a sodium channel (Nav1.5) that is highly specific to denervation in adult muscle [17]. For this reason, we examined the abundance of fibers ≤ 1000 μm^2 from in the aging rats and a size representing an equivalent percentile of the WT mice (≤ 575μm^2) in Sarco mice. In the very old rats, there was a nearly greater than 20% prevalence of these very small fibers. Sarco mice conversely had only 6% of these fibers **(Fig. 1A, B)**. Since we expect that Sarco mice are subject to repeating cycles of denervation and reinnervation consequent to NMJ instability secondary to reduced MuSK activation, the greater abundance of very small fibers in very old rat muscle could be indicative of a failure of reinnervation in advanced age as opposed to faulty agrin-MuSK signaling.

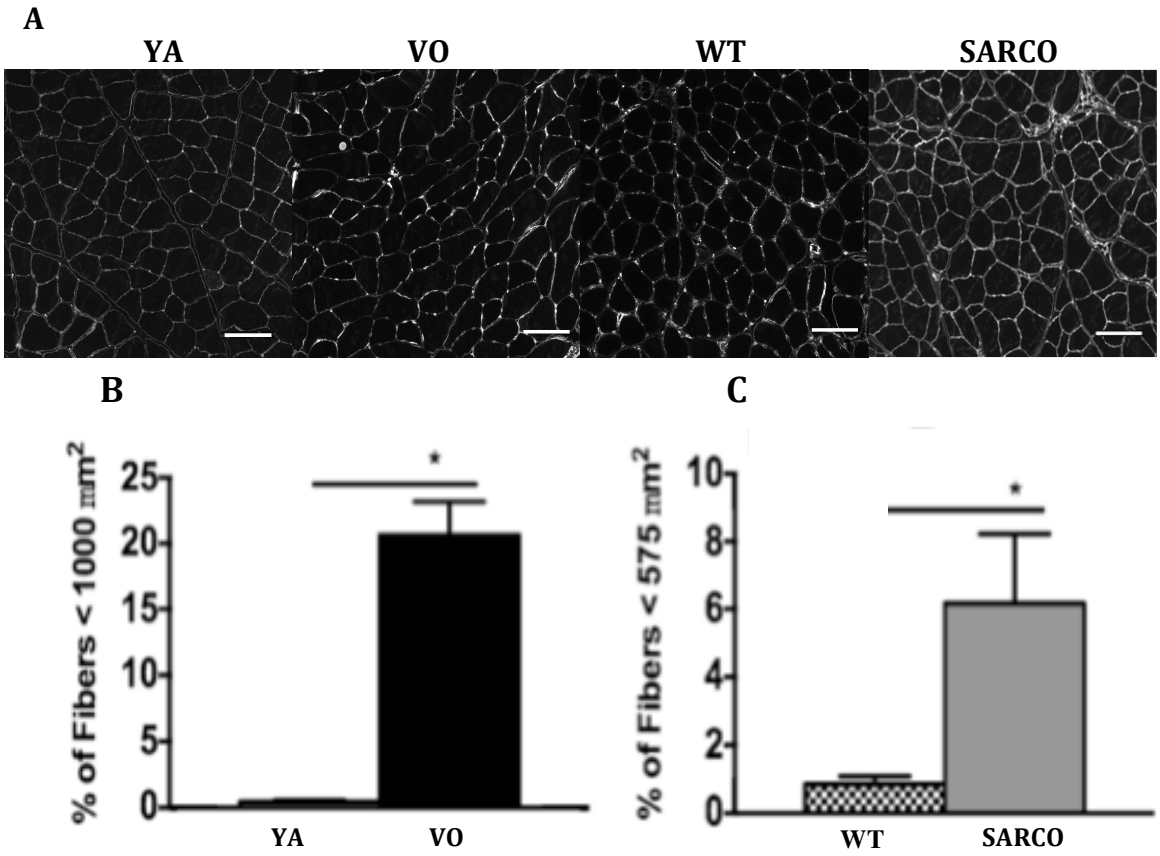


Figure 1. Small fiber prevalence in VO and SARCO is a morphological marker of denervation and mass loss. A) Representative images of fiber tracings in young adult (YA), very old (VO), wild type (WT) and Sarco mice (SARCO). Transverse sections from rat *Vastus Lateralis (*VL*)* and Sarco mouse *soleus (Sol)* were labeled with laminin in order to visualize the basal lamina and fiber outlines. Scale bar = 100 μm. B, C) Quantification of the proportion of fibers below a 'very small' fiber size threshold in Very old rats (*B*) and Sarco mice (*C*). B) Transverse sections of rat VL were labeled for AChRs using Alexa 488 conjugated α-bungarotoxin to locate NMJs. The smallest 1% of fibers from the YA group defined here are <1000μm in diameter. Nearly 20% of fibers in the VO group fell into this size range. C) In the sarcomice, we made a similar percentage based estimate resulting in a 575μm cutoff. There was a 6% prevalence of fibers of the same group in the Sarco mice. Quantification of fiber size was done using a two-tailed t test. Young adult: n = 8; Very old: n = 9; Wild Type: n = 3; Sarco mice: n = 5. Data are presented as means ± SE**.** * $P < 0.05$.

3.3.3 AChR Cluster Integrity

NMJ morphology can be used to assess the degree of NMJ instability [39, 60, 215] and has been well characterized in age and various muscle diseases [2, 61, 123, 261]. The morphology of the post-synaptic AChR clusters were determined visually by counting the number of segments appearing around the edge of muscle fiber cross-sections via semi-automated outlining on imageJ. We defined the AChR cluster integrity ratio as the number of NMJs exhibiting a single segment divided by the number of NMJs

exhibiting more than a single segment for each animal. A lower ratio signifies more NMJ fragmentation and a decreased NMJ stability. As can be seen in **Fig. 2A**, VO rats had a lower AChR cluster integrity compared to YA rats meaning they had a greater number of fragmented junctions. Similarly, the AChR cluster integrity index for Sarco mice was less than that of the WT mice (**Fig. 2B)**. As such, our results are largely consistent with prior studies showing AChR cluster fragmentation with aging [32, 39, 60, 184, 259] and neurotrypsin over-expression[39].

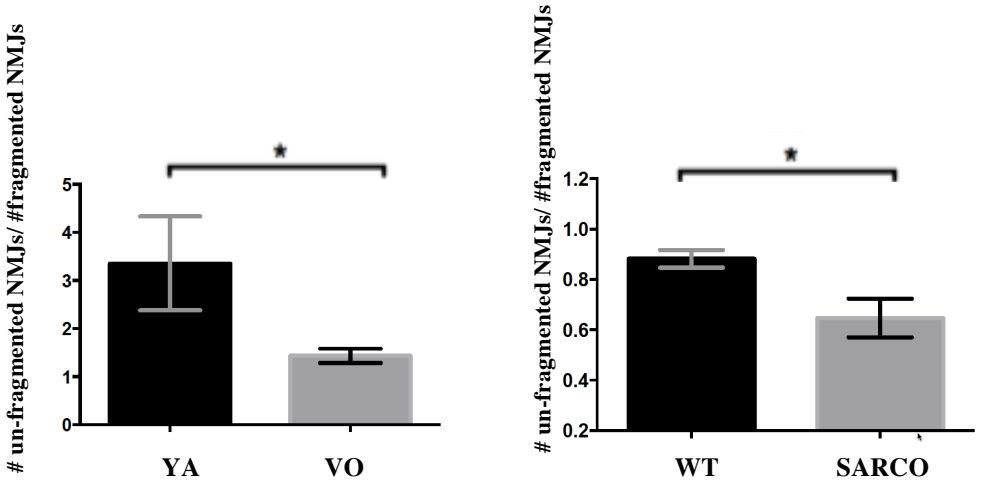


Figure 2. Increased fragmentation of the NMJ in both VO and SARCO compared to YA and WT, respectively. NMJ clusters were determined on Alexa 488 conjugated, α-bungarotoxin-labeled NMJs. Each fragment was separately outlined and counted. Each animal then had its number of non-fragmented NMJs divided by the number of fragmented NMJs, as such the sample size was determined by the total ratio averaged per each animal, not by the total number of junctions averaged per group. The larger the ratio, the less relative fragmentation was said to occur. A) We compared VO and YA rats, where YA rats had a greater (3.354 ± 0.9770, n=8) AChR cluster integrity ratio compared to VO rats (1.433 ± 0.1495, n=9). B) The AChR cluster integrity index for WT mice was greater (0.8821 ± 0.03532, n=3) than that of the SARCO mice (0.6471 ± 0.07658, n=5). Data are presented as means ± SD. * $P < 0.05$.

3.3.4 Protein Intensity levels at the Neuromuscular Junction

3.3.4.1 Protein Intensity levels at the Neuromuscular Junction In Sarco Mice

Given that the Sarco mouse is a model of sporadic denervation, we wanted to know if this model could explain the changes in intensities of the NMJ components. We first analyzed the protein intensities of WT and SARCO NMJs to gauge the impact of reduced MuSK activation on rapsyn and AChR at the NMJ and then compared this data

to that of the aging rats. Any changes or similarities were reasoned to indicate areas at the aging NMJ that were likely affected by faulty Agrin-MuSK signaling. Rapsyn intensity decreased by 11% in SARCO compared to WT animals (**Fig. 3B**), AChR intensity decreased by 25% in SARCO compared to WT animals (**Fig 3C**)**,** and MuSK intensity decreased by 44% in SARCO compared to WT animals (**Fig. 3D**). The decreases in the Sarco mouse NMJ intensities were directly attributable to the decreased agrin signaling due to neurotrypsin overexpression.

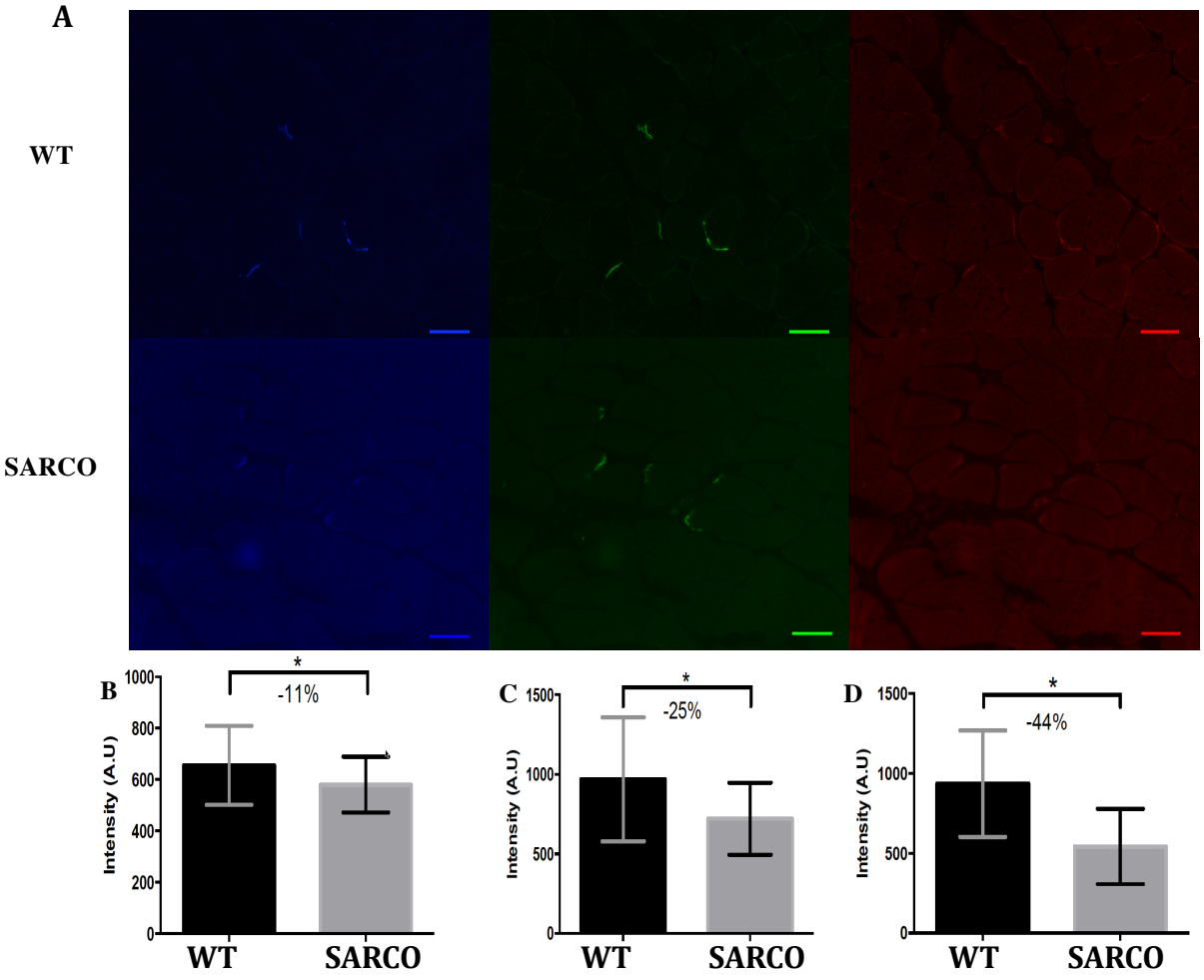


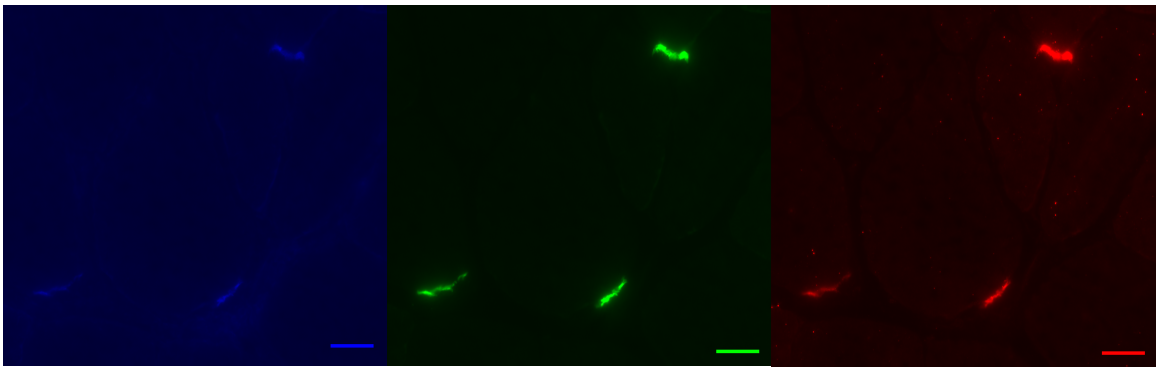
Figure 3. Intensity readings in WT v SARCO animals. *A)* The area around the NMJs on the Rapsyn (blue), AChR (green), and MuSK (red) channels were measured semi-automatically using a wand tool in imageJ. We measured intensities separately and obtained values for each channel in Sarco mice (n=296) and WT mice (n=185). Scale bar = 50µm. B) Rapsyn intensity decreased by 11% (580.8±108.8 AU) in SARCO compared to WT (655.6 ±153.6 AU) animals**.** C) AChR intensity decreased by 25% (721± 225.7 AU) in SARCO compared to WT (968.3 ±389.4 AU) animals**.** D) MuSK intensity decreased by 44% (542.7±236.3 AU) in SARCO compared to WT (936.3± 333.5 AU) animals..Data are presented as means ± SD. * $P < 0.05$.

3.3.4.2 Protein Intensity levels at the Neuromuscular Junction In Aging Rats

To investigate what could be precipitating the fragmentation of the NMJ in age we looked also looked at the Agrin-MuSK pathway in our F344 BN aging rat model. Given that AChR clustering is regulated in part by agrin-dependent MuSK activation[176, 262], we assessed whether there were any relative differences between the NMJ proteins of the agrin-signaling impaired Sarco mouse model and the aging rat model. Musk intensity decreased 34% in VO compared to YA animals **(Fig. 4D),** while rapsyn intensity increased 18% in VO compared to YA animals **(Fig. 4B)**. Despite the higher rapsyn levels AChR intensity still decreased, but only by 10% in VO compared to YA animals **(Fig. 4C)**. As such, whereas the decrease in MuSK is similar to what was seen in Sarco mice, downstream of this the changes were notably different with aging. The increase in rapsyn intensity with aging may suggest an attempt to stabilize the AChR clusters in response to denervation. Given that MuSK recruits rapsyn to the NMJ and there is a decreased MuSK in age, it is also possible that there is an increase in the amount of activated MuSK at the aging NMJ, but we did not assess this in our studies. Importantly, when compared to the changes seen in the Sarco mice, this also indicates that the Agrin-MuSK pathway is only mildly affected or even relatively maintained in age.

A

YA



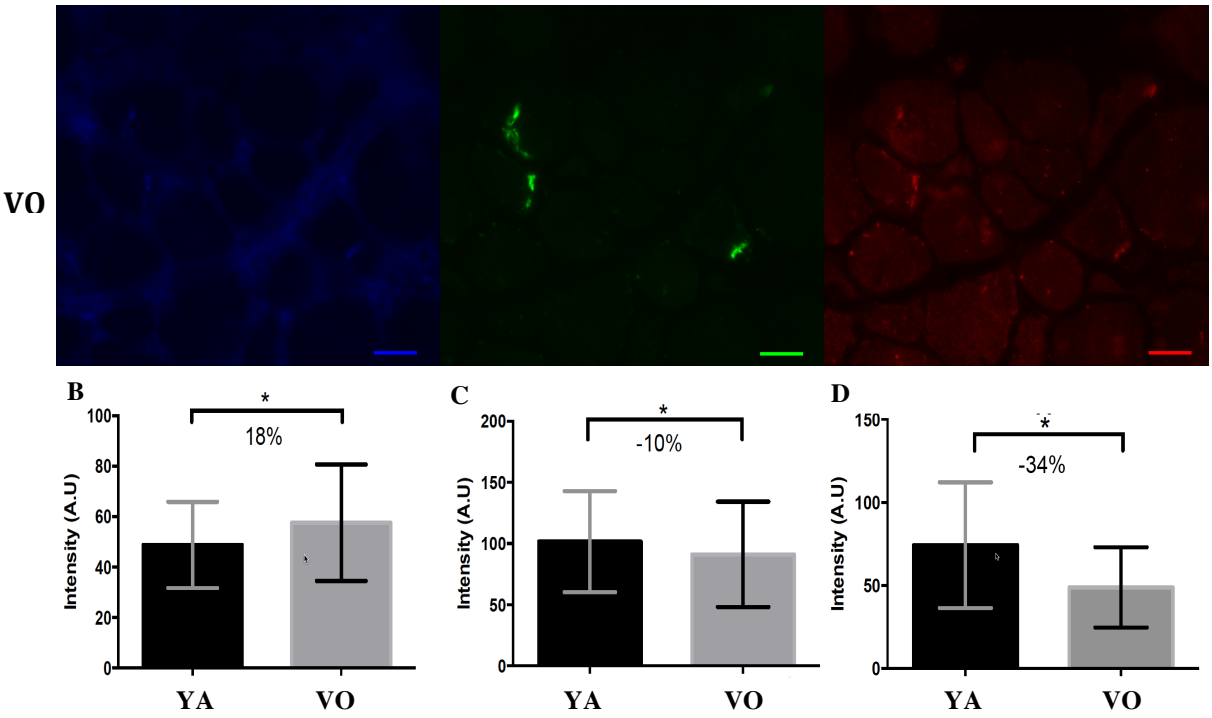


Figure 4. NMJ protein intensity readings of YA and VO Rats. *A)* We measured, Rapsyn (blue), AChR (green) and MuSK (Red) intensities separately and obtained values for each NMJ in YA (n=164) and VO (n=249) rats. Scale bars=25µm. The NMJ shape was determined via the wand tool in imageJ. A) Rapsyn intensity increased by 18% (57.65±23.07 AU) in VO compared to YA (48.81±17.06 AU p< 0.05) animals. B) AChR intensity decreased by 10% (91.27 ± 43.03 AU) in VO compared to YA (101.6 ±41.35 p<0.05) animals. C) MuSK average pixel value in the outlined region decreased by 34%(48.85 ±24.19 AU) in VO compared to YA (74.30 ±37.9 AU p<0.05) animals. Data are presented as means ± SD. * *P < 0.05*.

3.3.5 Rapsyn to AChR intensity Ratio at the NMJ

Given the primary difference in NMJ intensities between the two models was a differential direction of change in rapsyn intensity we suspected that this could be involved in maintaining NMJ stability in the old animals. Thus we wanted to look at rapsyn behavior more closely in the old animals by assessing rapsyn:AChR. This measure was used to indicate the degree to which AChR are immobile within the NMJ where we expect that a higher rapsyn:AChR indicates a more stable endplate[36, 263]. Rapsyn:AChR increased by 38% in VO compared to YA **(Fig. 5A)**. Rapsyn:AChR increased by 15% SARCO compared to WT **(Fig. 5B).** The reasons for these ratio increases were different in aging versus neurotrypsin over-expression. In the aging rat, the increase was due to an increase in rapsyn and smaller relative decrease in AChR

intensity compared to the Sarco mouse. This ratio increase with aging likely represents a true increase in AChR stability as the AChR is relatively maintained compared to Sarco mice. In the Sarco mice the increase in this ratio was driven by a large relative decrease in AChR intensity at the endplate. It may be that the large drop in the Sarco mice AChR reflects the large decline in MuSK activation and a subsequent preferential loss of AChR. In the aging animals it is possible that there is an external source of agrin (perhaps Peripheral Schwann cells) maintaining the activated MuSK and therefore rapsyn signal.

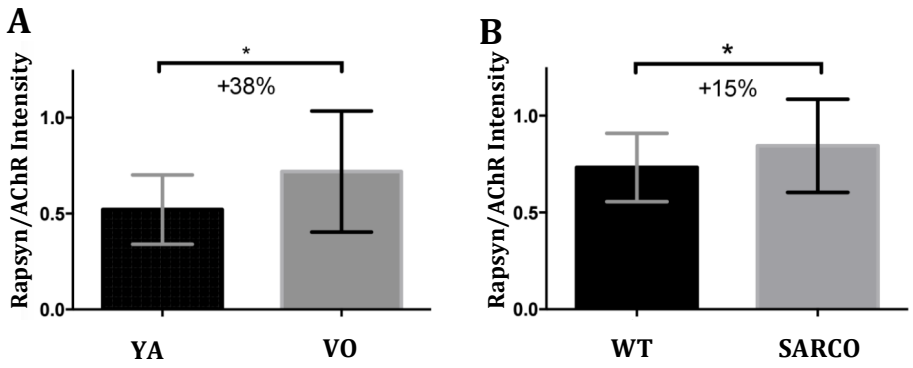


Figure 5. Rapsyn:AChR intensities at the NMJs of aging and transgenic animals**.** *A, B)* Rapsyn and AChR intensities were matched at each NMJ and calculated as Rapsyn intensity/AChR intensity. Values were previously determined by outlining the NMJs on each channel. *A)* A 38% increase in Rapsyn:AChR occurred in VO 0.719 ±0.3156,) compared to YA (0.5199 ±0.1810,). *B)* Rapsyn:AChR intensity increased in SARCO (0.8442±0.2410) compared to WT (0.7325±0.1766, $p<0.05$). Data are presented as means ± SD. * $P < 0.05$.

3.4 Discussion

There is widespread muscle dysfunction and atrophy in age. At the most clinically relevant ages (≥80 years old) persistently denervated fibers become interspersed throughout muscle and account for the majority of muscle loss in age [17, 78]. One proposed mechanism for persistent denervation is a disturbance in the signaling network regulating the post-synaptic NMJ structures. While the regulation of the NMJ has been

well characterized in development[35], there are still gaps of information of how NMJ changes may result in muscle dysfunction in very old age, despite reviews on the matter [2, 261]. Considering that NMJ dysfunction has been shown to occur in some circumstances following nerve damage and in other circumstances shown to occur independent of nerve damage, the extent to which NMJ changes with aging represent a cause versus consequence of motor neuron or motor axonal degeneration remains unclear[3, 61, 189, 264]. For this reason, we focused the current study on identifying whether NMJ dysfunction could account for persistently denervated fibers in very old age. In particular, we wished to evaluate the hypothesis that the agrin-MuSK pathway is disrupted in aging and contributes to NMJ dysfunction. We started by assessing the morphological changes in aging muscle and in a model of NMJ instability due to reduced MuSK activation (the Sarco mouse), and then determined whether the changes in the Agrin-MuSK pathway with aging were similar to what was seen in the model of impaired MuSK activation. Similar changes would be considered as evidence in favor of alterations in the agrin-MuSK pathway playing a role in aging.

3.4.1 Impact of Aging on Fiber Size Distribution

Studies have previously shown that between late middle age and very old age there is a significant increase in the number of very small fibers in both humans and rats [87, 104, 118]. This critical shift underlies many of the clinically relevant aspects of mass loss with age and may be a significant contributor to increased risk of frailty and general mobility impairment in very old age[45].

Studies conducted by Lexell et al. in the 1980's characterized muscle loss in humans aged 30 y compared to 70 y of age found that among various muscle groups,

there was a reduced muscle fiber number and CSA [265]. Lexell's group followed up the study by looking at men aged 13 to 83 y and found that muscle volume and number loss begin as early as 25 years old and progresses steadily thereafter[118]. Our other work has since shown that the majority of the very smallest fibers in advanced aged muscle are denervated [17]. Furthermore, denervation has been shown to increase the likelihood that mitochondria open their permeability transition pore (mPTP), which would in theory facilitate cell death and fiber loss of denervated muscle fibers [236]. These changes suggest that denervation plays a central role in both fiber atrophy and even total fiber loss seen in earlier studies. Additionally, an analysis of fiber size distribution in both *soleus* (a largely slow twitch muscle) and *gastrocnemius* muscle (a largely fast twitch muscle) in aging rat models has shown that a downshift in fiber size distribution is not fiber-type dependent[27]. The significance of these findings is that there is likely a common mechanism among all fiber types leading to severe atrophy in advanced age: denervation.

To gain insight into the prevalence of fiber atrophy among our aging and Sarco mouse models we measured fiber size distribution using previously established criteria that helped us determine which fibers were normal and which were very small. A previous study examining long-term denervated fibers caused by surgical nerve transection revealed that 2 months post treatment, 90% of fibers shrank to ≤ 1000μm^2[266]. An assessment by our lab showed that the vast majority (>90%) of fibers ≤ 1000 μm^2 (very small fibers) in very old muscle express $NaV_{1.5}$, a marker of denervation[17]. For this reason, in aging muscle we examined the amount of fibers ≤ 1000 μm^2, as these represent a population of fibers that are in very low abundance in young adult muscle (1 percentile) and were most likely to be denervated. In the Sarco

mice we used 575μm^2 as a cutoff, based upon this representing the 1 percentile size of wild type mice (same percentile cut-off as in aging rat). Our results indicate that whereas there was a greater than 20-fold increase in small fibers in VO compared to YA, the SARCO showed a 6-fold increase in small fibers compared to WT muscle. The stark difference between the increases in each group relative to their controls suggests that either the burden of denervation events is greater with aging or that the capacity for reinnervation fails in advanced age. With respect to denervation, in our current study we next wished to understand the degree to which the large accumulation of denervated muscle fibers was associated with neuromuscular junction fragmentation, as will be discussed below.

3.4.2 Changes in Neuromuscular Junction Morphology

The first evidence of NMJ deterioration with aging was reported in rats in 1966[56] and was confirmed to occur in aging humans two decades later[257]. These and other studies characterized the AChR clusters on the myofiber side of the NMJ as having a "pretzel'-like appearance, which has been shown to degenerate into a "fragmented" pattern in both age[60, 61] and in neuromuscular diseases like muscular dystrophy[194, 267, 268], slow channel syndrome[191, 269], myasthenia gravis[53, 238, 270] and ALS[189, 229]. NMJ fragmentation has been shown to occur immediately after the denervation and reinnervation events, which are central to age-related physiological changes like fiber atrophy and decreased reinnervation potential[17, 61, 160, 162]. Fragmented NMJs themselves indicate general NMJ instability and lead to reduced ACh conduction efficiency [154] and decreased muscle fiber conduction velocity[168].

Therefore, understanding the mechanism by which clusters fragment is important for interpreting how NMJ instability affects the neuromuscular environment.

We explored whether NMJ destabilization could account for the increase in persistently denervated fibers by first assessing the degree to which the NMJs were fragmented. We quantified fragmentation by measuring the ratio of fragmented to non-fragmented junctions, which we termed the 'fragmentation index'. In addition to the increase in the amount of small fibers, we found that there was an increase in the fragmentation index of both Sarco mice and aged rats. One limitation of our measurement was that the fragmentation assessment was done in muscle cross-sections, which gave us an incomplete view of the total neuromuscular junction architecture on the muscle fiber longitudinal surface. As such, it was necessary that we average the fragmentation results over all of the NMJs of a given animal to gain a sense of an estimated amount of fragmentation overall. Identification of individual fragmented NMJs was thus not possible with our approach. Notwithstanding, our data is in agreement with the literature, which has shown that there is an age-related increase in NMJ fragmentation [18, 27, 30, 123, 256, 264]. In the ageing rats many small and denervated fibers indicated that a large amount of persistent denervation had accrued throughout the animals' lifetimes. This is consistent with the observations that have related a simultaneous increase in denervation and fragmentation in age[32, 39, 60, 184, 259]. In the Sarco mice fragmentation was directly attributable to the decrease in agrin signaling and subsequent decrease in MuSK activation, which has been shown to contribute to NMJ instability[39]. Sarco mice have been shown to indicate signs of NMJ deterioration in addition to a variety of other physiological alterations like fiber type grouping and fiber loss[39]. These findings

confirm that both long term denervation in aging rats and the sporadic denervation occurring in the Sarco mice can lead to NMJ fragmentation. These also suggest that sporadic denervation is sufficient to cause NMJ fragmentation but may not lead to a large accumulation of very small and persistently denervated fibers if reinnervation occurs relatively quickly. Regardless of this point, we next we wanted to know whether changes in the Agrin-MuSK pathway could account for the fragmentation we saw in ageing rats by comparing changes in the agrin-MuSK signaling pathway to what was seen in our model of impaired MuSK activation (Sarco mouse).

3.4.3 Impact of Aging on MuSK, AChR and Rapsyn Protein Levels at the Neuromuscular Junction

Whereas denervation of muscle fibers is a normal occurrence in adulthood, such fibers are usually successfully reinnervated and this leads to the well-known fiber type-grouping seen in aging muscle. In contrast, it is unknown what causes a subset of denervated fibers to remain denervated over long periods of time in very advanced age, a phenomenon that leads to escalating fiber atrophy and angularity[119]. One possibility is that there is aberrant signaling in the pathways responsible for maintenance and/or restoration of neuromuscular junction stability.

The focal point of this study was to assess the hypothesis that NMJ instability in age is due to changes in Agrin-MuSK signalling. As previously mentioned, the isoform z-agrin (which we have been referring to simply as agrin herein) from the motor neuron maintains the integrity of the NMJ by phosphorylating and activating MuSK through its co-receptor LRP4 [35, 38, 175, 176]. In the absence of neural agrin, the adult NMJ disassembles within days [37, 38]. Agrin is also critical for AChR clustering during

development [144]. Activated MuSK then interacts with Dok7, in turn enabling MuSK phosphorylation and subsequent auto-activation, a sequence required for AChR clustering during NMJ maintenance and development [177]. Beyond MuSK mediated AChR density control, Agrin acts to stabilize the AChR lipid domains through an effector called rapsyn. Agrin interacts with rapsyn through Adenomatous Polyposis Coli (APC), and both slows rapsyn kinetics and anchors the AChR to the cytoskeleton into groups of AChR lipid rafts [35, 179]. There are a number of agrin signalling regulators that were not explored in this study but are nonetheless important, including MuSK endocytosis[180] and lipid microdomains [33]. We briefly touched on the effect of intracellular calcium [181] on the potential interaction with the agrin-MuSK pathway and remodelling on the NMJ in section 2.4.2.2. Since the agrin-MuSK pathway is key in maintenance of the NMJ and its response to denervation, studies have recently explored whether this pathway is changed in age and in neuromuscular diseases [2, 31, 39]. Our study employed the use of the Sarco mouse which allowed us to identify the impact of a specific defect in Agrin-MuSK signalling on downstream protein levels including AChR density and rapsyn and compare this to what occurs in aging rat muscle.

When we analyzed Rapsyn, AChR and MuSK protein intensities at the NMJs of Sarco mice, all intensity levels decreased. In contrast, whilst reductions in MuSK were similar in aging rat as in Sarco mouse muscle, rapsyn actually increased and the decline in AChR intensity was not as severe as in Sarco mouse. The AChR intensity decrease in both Sarco mouse and aging rat muscle is consistent with previous studies showing that MuSK activation is necessary for agrin-dependent AChR clustering [262, 271] and Rapsyn-AChR interaction[272]. On the other hand, the increase in rapsyn and smaller

decline in AChR intensity with aging suggests that the agrin-MuSK pathway may be relatively maintained in aging muscle and is thus, unlikely to be the cause of the NMJ fragmentation and persistent denervation we observed.

In the context of other studies examining reasons for NMJ fragmentation with aging, a repeated intravital observation of the same NMJs over a 2-6 month period revealed that fragmentation occurs suddenly in 16-24 month old mouse *sternomastoid* muscle[61]. Changes in AChR intensity were shown to follow sudden fragmentation events, therefore as the NMJ ages, necrosis and the ensuing fiber basal laminal infolding that occurs during regeneration remodels the NMJ to exhibit a punctate AChR pattern[61]. As MuSK signaling is required to anchor AChRs to the cytoskeleton via Rapsyn[262, 271] the persistence of low MuSK levels in aged NMJs could render these fibers more vulnerable to atrophy secondary to failed reinnervation[155]. In contrast to fragmentation Li and Thompson's conclusions, average fragmentation patterns have been shown to increase gradually over time in diaphragm and other muscles with aging [32], suggesting that NMJ damage does not have to be sudden or that mechanisms of fragmentation may vary to include fiber damage or motor neuron damage over an organism's lifetime. Interestingly, Rudolf reviewed the matter and intuited that the comparatively short lifespan of the rodent models may affect our interpretation of a sudden versus gradual sequence of events. That is, minor age-related rearrangements like gradual AChR fragmentation or molecular rearrangements imaged once every few days could be missed using our instrumentation whereas these events could be more gradual in humans[2]. This idea has not been explored and as such assessing the time course of NMJ instability in a longer-lived organism would be an interesting area of inquiry. It has been

proposed that one determinant of whether an NMJ accumulates damage over time is determined by the type of nerve (spinal or cranial-derived) innervating the NMJ [189] suggesting that motor neurons factor heavily in determining NMJ stability over time. Taken together, while fiber damage has been suggested to accrue in muscles over time there may also be simultaneous neural damage, which determines the degree to which an NMJ is susceptible to fragmentation.

We suspect that the rapsyn increases in the aged rats are a response to denervation and effectively slow AChR turnover. This idea is supported by the increase in Rapsyn:AChR in very old animals. Like with AChR integrity ratio, the Rapsyn: AChR intensity represents the stability of the NMJ[263]. Unlike the AChR fragmentation index, the Rapsyn:AChR indicates the future likelihood of AChR migration [273, 274]. A Higher Rapsyn:AChR indicates a decreased likelihood of AChR migration from the junction. In our study, both the VO and SARCO animals showed an increase in Rapsyn:AChR compared to YA and WT, respectively. One study showed that when Rapsyn conjugated to enhanced green fluorescent protein (Rapsyn-EGFP) occupies extra-synaptic binding sites, the endplate is further stabilized without any apparent change in the AChR packing density[263]. This suggests that the increase in the Rapsyn:AChR and the relatively low decrease of AChR at the very old compared to Sarco mouse NMJ may reflect a sufficient rescue of the density to maintain stability at the NMJ. Ideally, more experiments assessing the kinetics of Rapsyn and AChR at the aging NMJ and after a forced NMJ destabilizing event, such as after partial nerve crush, should be done to fully answer this question.

One possible explanation for how the aging endplate is able to maintain agrin signaling is that Peripheral Schwann Cells (PSCs) are stimulated to maintain agrin signaling in the aged animals. PSCs sense a nerve pullback and release agrin in order to attract neighboring motor neurons to the denervated muscle[274, 275]. Partial denervation has been shown to stimulate the growth of PSCs to 'envelope' the synaptic space [276]. This could have the effect of increasing agrin availability at the endplate, resulting in an increased Rapsyn:AChR via rapsyn maintenance.

3.4.4 No Precedence for AChR and Rapsyn levels Changing in Opposite Directions

Normally, there is a tight interaction between rapsyn by AChR, although these affinities have been known to change, for instance, when rapsyn is associated with extrasynaptic AChR [277, 278]. To our knowledge there is no precedence for an observed different direction in AChR and rapsyn intensities in either age or neuromuscular disease models. However, a comprehensive genomic study of aging NMJs indicated that while the expression levels of AChR went up significantly, there was no change in rapsyn expression[279], perhaps indicating a point of regulation bifurcating at transcription. Additonally, it has been shown that rapsyn and AChR can be shuttled to the membrane independently of one another and rapsyn can be present in the membrane at different concentrations[280]. Rapsy: AChR stoichiometry can range from 1:1-3:1 depending on the on the AChR subunit composition [278]. Given that different AChR subunits are expressed differently due to innervation status, it follows that while AChR with lower rapsyn affinity may repopulate the NMJ, rapsyn may not be regulated in tandem with AChR. Furthermore it should be noted that Rapsyn can self-associate yet it is unknown us

us whether post-translational modifications in age can induce competition between rapsyn-AchR / rapsyn-rapsyn interactions.

3.4.5 Possible Neural involvement in Age related NMJ Instability

As discussed above, we observed several differences in aging rat muscle from what is seen in our mouse model of reduced MuSK activation, suggesting impaired MuSK signaling is unlikely to be the cause of NMJ fragmentation and persistent denervation in aging muscle. Given that spinal motor neuron cell bodies have been shown to decrease in age[70], and we have shown that this also occurred in the rats studied here[17], but are maintained in the Sarco mice[39], we suspect that motor neuron loss may be the primary cause of denervation in aging. Therefore the modest changes we observed in the Agrin-MuSK pathway with aging are possibly a result of neurally driven denervation. To assess this more directly, in the future we would like to repeat these experiments and include a model with a specific pre-synaptic motor neuron defect. One of these models could be an inducible knockout of the Survival Motor Neuron (SMN) gene in a mouse model called SMNΔ7 SMA. This model approximates a motor neuron disorder called spinal muscular atrophy (SMA), which shares many phenotypes with aged muscle. Interestingly, analyses using these mice showed that mature NMJs without SMN had a relatively mild effect. However, selective NMJ pathologies accrued in injured and aged animals only [281], suggesting that NMJ dysfunction may only present itself when secondary failures of NMJ maintenance accumulate in old age. Interestingly, a side by side analysis of SMA and ALS mice, another potential model with a primary motor neuron defect, revealed that whereas pre and post-synaptic defects occurred

simultaneously in SMA deficient mice, pre-synaptic alterations in ALS mice preceded post synaptic alterations[282]. It should be noted that given the amount of similarities regarding the trajectory and phenotypic seen between aging muscle and ALS, the fact that fragmentation in ALS accumulates after nerve defects challenges the idea that NMJ fragmentation in age is solely due to sudden trauma in muscle cells[61].

3.4.4.1 Varied Models of NMJ Deterioration Recapitulate Aging Phenotypes

In addition to SMNΔ7 SMA and ALS SOD^{G93A} mice, numerous other mouse models have been used to examine the effects of pre and post-synaptic defects on muscle mass in both disease and age. The defects due to denervation have been outlined through the use of SOD1 knock out[190], TrkB heterozygous knockout[41] and ATG7 knockout mice[43]. These models all have unique defects but result in many similar phenotypes, which include fiber type grouping, fiber type co-expression, NMJ fragmentation and fiber atrophy[117]. Through increased oxidative stress, the $SOD1^{-/-}$ mouse has neuromuscular junction instability and motor neuron dysfunction[215, 283, 284]. The $ATG7^{-/-}$ knockout mouse has a defect in autophagy that results in faulty AChR recycling[43]. The $TrkB^{+/-}$ knockdown mouse has a reduced number of muscle receptors for BDNF and neurotrophin 4/5 [41] resulting in an impaired neurotrophic response. The $LMNA^{-/-}$ Laminin A-null mouse model has a disrupted nuclear laminin gene which results in disrupted myelination and other motor axonopathic features[285]. As previously mentioned, there are also endplate –affected models, which recapitulate the phenotypes in the aging. The spinal muscular atrophy model with the survival motor neuron gene knockout has disrupted NMJs as well as disrupted motor neurons[286, 287]. Models of ALS like the SOD^{G93A} mouse have accelerated denervation as well as NMJ

instability[190, 288]. While all of the aforementioned models have very distinct sources of neuromuscular dysfunction, many of them lead to very similar phenotypes that strongly resemble those occurring with aging. This poses a significant challenge in that the muscle traits seen with aging are not specific to a single cause and thus, identifying which mechanisms are causing NMJ destabilization and denervation with aging is extremely difficult. Indeed, it is possible that aging involves a multi-factorial and systemic failure that presents itself as the 'phenotypic suite' we observe as muscular aging. Notwithstanding this possibility, specific differences among these models when compared to age may help us determine which defects are more or less likely to contribute to the defects, which accumulate in age. In our study, use of the Fischer 344xBrown Norway F1-hybrid (F344BN) aging rat model and Sarco mouse model allowed us to determine which phenotypes in age could potentially originate from a specific Agrin-MuSK signalling defect. While many aforementioned models have been compared to age, this study is the first to make a direct comparison between the muscle fibers and NMJs of an aging model and the Sarco mouse model of impaired Agrin-MuSK signalling. Thus, our results showing some distinct changes with aging versus Sarco mice at a minimum allow us to rule our impairment in MuSK signalling as the major reason for NMJ instability and persistent denervation in aging muscle.

3.4.5 Limitations and Future Directions

There are a number of limitations to this study. First off, as previously mentioned the fragmentation data was collected in muscles in cross-section as opposed to longitudinal orientation. On-face images of the NMJs (parallel to the fiber longitudinal orientation) are the only reliable estimation of complete fragmentation in a single NMJ.

As such, we could not get an exact number of fragments per endplate and had to average the number of fragments over the cross sections of all endplates in an animal and then compare the average of the group to that of another group. This measurement also resulted in high animal to animal variability. Additionally, none of the measurements taken in this study factored in the potential fiber-type specific effects on the NMJ. We would have liked to measure the changes in the NMJ proteins on a fiber-type specific level, which may have yielded different results. For instance, a previous study noted that there were differences among fiber types of pre and post-synaptic morphology of aging NMJs in *soleus* and EDL muscles [289]. Fiber typing and staining for NMJs in serial sections could have either confirmed or challenged our findings that there was maintained signaling in age compared to Sarco mice in specific fiber types. We would have also liked to expand the study by including a presynaptic-defect model that would have allowed us to compare the changes in the NMJs between pre-synaptic defect (like the previously mentioned SMNΔ7 SMA model), post-synaptic defect (like the Sarco mouse), and aging models. This would have allowed us to take our analyses further and possibly allow us to more clearly conclude whether the changes in age are more post-synaptic or pre-synaptic in origin. Finally, a measurement of the amount of MuSK activation (for instance p-MuSK:total MuSK) would have allowed for a deeper analysis of the effect of age on the agrin-MuSK pathway. A relative maintenance in the amount of p-MuSK despite the small decrease in total MuSK would support the idea that the agrin-MuSK pathway is maintained in age.

3.4.6 Conclusions

Denervation is clearly involved in aging muscle atrophy and this makes it an attractive target for future research and investment [27]. Research has implicated NMJ instability as a probable cause of denervation in age and in turn NMJ instability makes it more difficult to reinnervate and likely precipitates fiber atrophy[2, 209]. Collectively, our results indicate that in spite of fiber loss and NMJ fragmentation, and an associated decline of MuSK at the NMJ, aging rats have slightly elevated rapsyn protein levels at the NMJs, suggesting impairment in the agrin-MuSK pathway is unlikely in aging muscle. This is supported by the comparatively small decrease in AChR intensity at the aging NMJ compared to the Sarco mouse. Future studies should aim to determine if the changes between the NMJ protein levels are related to a post-translational problem. The implications of these findings are that therapies for aging muscle should not focus on post-synaptic endplate agrin-MuSK maintenance per se. It would be more beneficial to treat the cause rather than the symptom of repeated denervation, as decreased MuSK activation may be secondary defect due to a primary event like motor neuron death.

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