EXERCISE-INDUCED SIGNALING IN SKELETAL MUSCLE OF SPINAL MUSCULAR ATROPHY MICE

ACUTE EXERCISE-INDUCED SIGNALING IN SKELETAL MUSCLE OF SPINAL MUSCULAR ATROPHY MICE

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Lay Abstract

Spinal muscular atrophy (SMA) is a health- and life-limiting neuromuscular disorder that affects every 1 in 10,000 live births. Chronic physical activity is therapeutic in SMA animals and patients, however the underlying mechanisms for these exercise training-induced adaptations have yet to be fully described. Examining these processes may reveal novel therapeutic targets for SMA patients, as well as increase our knowledge of the basic biology of this complex disorder. Thus, the purpose of this thesis was to investigate the cellular effects in skeletal muscle of a single bout of physical activity in a pre-clinical mouse model of SMA. We observed that molecules important for maintaining and remodeling the neuromuscular system were enhanced following exercise in SMA-like mice. Furthermore, activation of proteins that reduce the molecular dysfunction of SMA was also evoked following a single bout of physical activity. Our data identify novel cellular pathways in the exercise biology of SMA.

Abstract

Spinal muscular atrophy (SMA) is the leading genetic cause of infant mortality and second most prevalent autosomal recessive disorder. SMA is caused by mutations in the survival motor neuron 1 (SMN1) gene resulting in the deficiency of the crucial survival motor neuron protein (SMN). Prescribed physical activity is an emerging therapy for this disorder, however the cellular and molecular mechanisms of exercise in SMA have yet to be fully elucidated. Examining the exercise biology of SMA may prompt the discovery of novel and effective therapeutic avenues for this pathology. Hence, we sought to determine the effects of a single bout of physical activity on intracellular signaling cascades and SMN expression in the skeletal muscle of SMA-like animals. AMP-activated protein kinase (AMPK) and p38 mitogen-activated kinase (p38) expression and activity were unchanged at pre-, early-, and late-symptomatic stages of *Smn*$^{2B/-}$ mice, which suggests that important molecular machinery for driving exercise adaptations were preserved. We then subjected *Smn*$^{2B/-}$ animals to an acute, endurance-based exercise protocol and collected skeletal muscle tissue immediately after or 3 hours post-exercise. Physical activity elicited significant activation of the AMPK-p38-peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) axis in *Smn*$^{2B/-}$ animals, which confirmed the preservation of canonical exercise-induced signaling in the SMA-like condition. Exercise also elicited alterations in the activation of protein kinase B (AKT), extracellular signal-regulated kinase (ERK), and ETS-like gene 1 (ELK). Collectively, these exercise-induced changes in the AMPK-p38-PGC-1α and AKT/ERK/ELK cascades occurred coincident with enhanced SMN expression. Lastly, acute exercise resulted in the normalization of autophagic signaling, indicating that physical activity may serve a novel role in correcting the aberrant autophagy program in SMA. In summary, this study expands our knowledge

of the molecular mechanisms of exercise biology in SMA and identifies the AMPK-p38-PGC-1α signaling axis as a potential regulator of SMN expression.

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List of Abbreviations

AAV – Adeno-associated virus

ADP – Adenosine diphosphate

AKT – Protein kinase B

αMN – α-motoneuron

AMPK – AMP-activated protein kinase

ARE – AU-rich elements

ASO – Antisense oligonucleiotide

ATG14 – autophagy-related gene 14

AU – Adenylate and uridylate

BNIP3 – BCl2/adenovirus EIB 19kda

bp – Base pair

Ca^{2+} - Calcium

CaMKII – Ca^{2+}/calmodulin-dependent kinase II

CREB – cAMP response element-binding protein

ELK – E26 transformation-specific domain-containing protein

ERK – Extracellular regulated kinase

Gabrapl1 – GABA receptor-associated protein-like 1

HDAC – Histone deacetylase

hnRNP – heterogeneous nuclear ribonucleoprotein

HuR – Human antigen R

IGF-1 – Insulin growth factor-1

IGF-1R – insulin growth factor 1 receptor

JAK – janus kinase

kDa – Kilodalton

KSRP – K-homology splicing regulatory protein

LC3 – Microtubule-associated protein 1A/1B light chain 3

MAPK – Mitogen-activated protein kinase

MEF2 – Myocyte enhancing factor 2

NMD – Neuromuscular disorders

NMDAR – N-methyl-D-aspartate receptor

NMJ – Neuromuscular junction

NR2A – GluR epsilon 1

p38 – p38 mitogen-activated protein kinase

p62 – p62/sequestosome-1

PGC-1α – Peroxisome proliferator-activated receptor gamma coactivator-1α

PI3K – Phosphatidylinositol-3 kinase

RBP – RNA binding protein

SMA– Spinal muscular atrophy

SMN – Survival motor neuron

SNAP25 – Synaptosomal-associated protein 25

snRNP – small nuclear ribonuclear proteins

STAT5 – signal transducer and activator of transcription 5

UBA1 – ubiquitin-like modifier activating enzyme

ULK1 – unc-51-like kinase 1

Declaration of Academic Achievement

Sean Ng was the principal contributor. Andrew Mikhail assisted with immunoblotting analysis. Vladimir Ljubicic assisted with conceiving and designing, as well as with writing the manuscript.

Review of the Literature

1. Introduction to spinal muscular atrophy

Spinal muscular atrophy (SMA) is a life-limiting neuromuscular disorder that affects 1 in 10,000 live births, making it the second most common autosomal recessive disorder. This disorder presents itself in several clinical forms, which are based on the phenotype and age of onset. The more severe variant of SMA, type 1, represents approximately 60% of all cases[1]. Patients with type 1 SMA have onset before 6 months of age, are unable to sit, and die prior to their second birthday. SMA patients with the milder forms of SMA, Type 2, 3, or 4, represent the remaining 40% of all SMA cases. Type 2 SMA patients experience disease onset at 7 to 18 months of age and experience difficulties with daily living tasks such as standing and swallowing. Children with type 3 and 4 SMA possess functional independence and typically are able to live to adulthood[1]. Managing these clinical symptoms of SMA requires substantial amounts of healthcare resources. On average, the economic cost of SMA is significant for patients and their caregivers (~$48,000/year), as well as for government health care systems (up to ~$500,000/per patient)[2]. The cardinal signs of SMA are the degeneration and death of α-motoneurons (αMNs) resulting in severe skeletal muscle wasting and weakness. Recent work, however, is now identifying SMA as a multisystem disorder affecting the cardiovascular, respiratory, digestive, and musculoskeletal systems[3]. For example, a spectrum of cardiac arrhythmias[4], vascular defects[4–6], and metabolic abnormalities[7] are common pathological features of SMA patients. These symptoms are likely to be secondary to the neuromuscular impairments but strongly contribute to the pathogenesis of SMA.

The majority of SMA cases are caused by mutations in the survival motor neuron (SMN) 1 gene and deficiency of the encoded product, SMN protein. The alteration of the SMN1 gene causes patients to depend on the virtually identical SMN2 gene to produce

SMN protein. However, a single base pair difference at exon 7 results in 80-90% of SMN transcripts to lack exon 7 resulting in a defective and rapidly degraded translation product. The remaining 10-20% of the transcribed SMN mRNA are full length and can be translated to functional SMN protein. SMA patients can possess multiple copies of the SMN2 gene where the disease severity of SMA is inversely correlated with the number of SMN2 copies they possess[8].

In order to comprehensively investigate the mechanisms and pathophysiology of SMA, several mouse models have been generated that recapitulate many aspects of the disease phenotype. While humans possess SMN1 and SMN2 genes, mice do not harbor the SMN2 gene. To address this, researchers genetically modify animals to replace the murine SMN gene with copies of the human SMN2 gene to exhibit a variety of SMA disease phenotypes. For example, a common murine model used to represent a severe type 1 SMA phenotype is the *Smn-/-; SMN2* mouse, which harbors a low copy number of SMN2[9,10]. SMA can also be represented without the SMN2 gene by altering the murine SMN gene. For example, the $Smn^{2B/-}$ murine model harbors a knock-in allele that interrupts SMN splicing, similar to the spicing patterns of human SMN2, and exhibits a less severe SMA phenotype with a lifespan of ~3 weeks[11].

2. The cellular function of SMN

The ubiquitously expressed SMN protein can be located in the cytoplasm, and nucleolus, as well as punctate nuclear structures called gemin coiled bodies and cajal bodies. SMN protein has also been linked to several cellular regulatory functions. For example, nuclear and cytosolic SMN has an essential role in the assembly of the small nuclear RNA, which encode for small nuclear ribonuclear proteins (snRNPs). snRNPs are essential for recognizing splicing sites and catalyzing intron removal in particular pre

mRNA[12]. Notably, SMA patients have been reported to possess a lower amount of snRNPs suggesting a reduced splicing capacity[13,14]. Cytosolic SMN protein has also been shown to be involved in the homeostasis of bioenergetics pathways and ubiquitin-driven mechanisms[15]. Furthermore, SMN can also influence healthy actin dynamics when present in the cytosol[16]. Indeed, cytosolic SMN binds to profilin permitting the release of Rho Kinase and downstream phosphorylation of cofilin and myosin light chain phosphatase[16]. Along these lines, neuronal SMN protein in the motor axon and synapse has been shown to serve as an important player in mRNA transport and synaptic vesicle release[17,18].

3. Requirement of SMN

Although SMN proteins can be found in all cell types, the expression and requirements of SMN are cell-specific. Cell types that demand high levels of SMN expression are associated with SMA pathogenesis as SMN is depleted to minimal levels. Neural tissues, such as the brain and spinal cord, are vulnerable to the pathology and require a large amount of SMN protein relative to other tissue types[19]. Similarly, lymphoid organs also require a considerable amount of SMN protein and have also been attributed to be an important contributor to the onset of SMA[20]. Other peripheral tissues, such as muscle, typically require lower levels of SMN but are still necessary for function and development[21–23].

It is important to note that the presence of SMN is essential for the development and maintenance of many tissues and organs. During early stages of life, sufficient levels of SMN are necessary for neuromuscular development[23,24], myogenesis, muscle differentiation[21,22], and immune system development[25]. Furthermore, the temporal difference in the development of the systems necessitates varying levels of cell-specific SMN protein at such time points. For example, high levels of αMN SMN protein are

required throughout the maturation of the neuromuscular junction (NMJ) [up to the postnatal day (P) 20], while the SMN requirement for muscle peaks at earlier stages (prior to P5)[26]. During post-developmental stages, the requirement for SMN drastically decreases to minimal amounts at basal states. Notably, the maintenance of particular cell types requires elevated levels of SMN protein. For example, when SMN levels of mature mice are depleted to minimal levels, the ability to repair and remodel the neuromuscular system is lost[23,24].

4. SMN regulation

Transcription and translation processes control the production of proteins while protein breakdown mechanisms such as the ubiquitin-proteasome system and autophagy regulate the protein degradation (Figure 1). On balance, these processes control the expression of proteins and possess multiple layers of regulation. Understanding these processes is important to highlight therapies that can enhance SMN gene expression and stability, as SMN is deficient in all forms of SMA. Hence, this section will discuss the mechanisms that regulate SMN levels.

i. Transcriptional regulators of SMN

SMN expression is mediated through a series of regulatory processes along the central dogma, including pre- and post-transcriptional modifications, as well as post-translational changes. The virtually identical SMN1 and SMN2 genes are regulated during cell growth and differentiation. Notably, the upstream promoter sites are common between these two genes[27]. These promoter sites permit the binding of important transcription factors that may encourage or repress the transcription of the SMN genes.

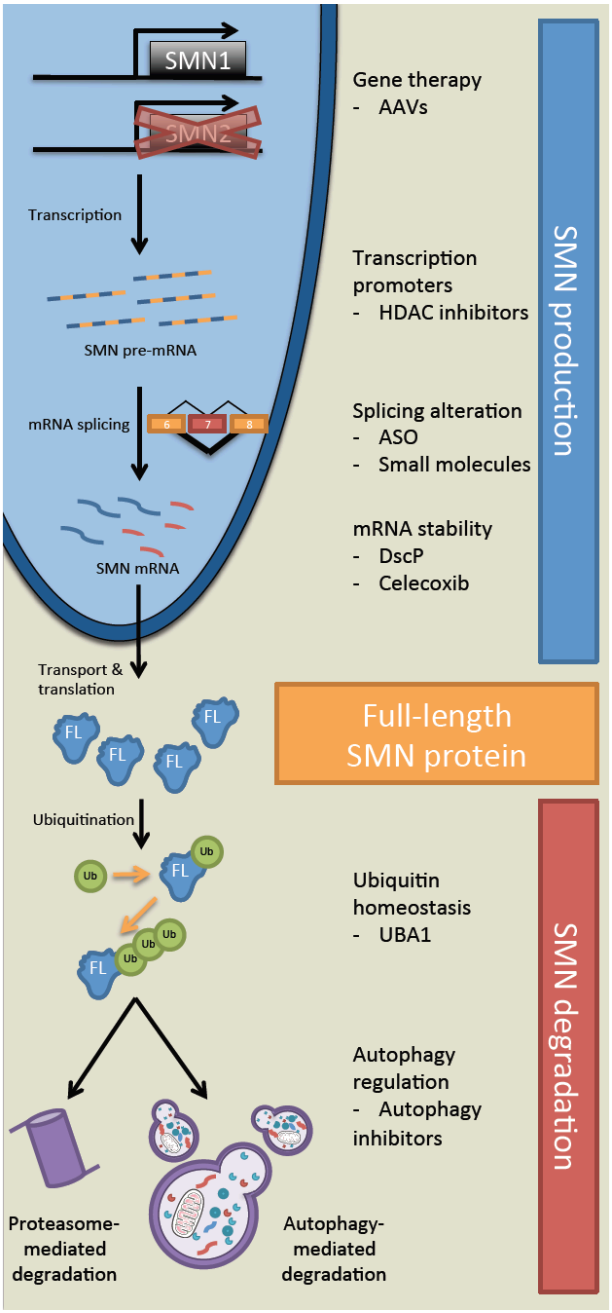

Figure 1. Regulatory mechanisms of survival motor neuron protein expression. Mutations in the SMN1 gene results in the deficiency of the survival motor neuron (SMN) protein. The abundance of full-length SMN protein is regulated, on balance, by various production and degradation mechanisms. Utilizing interventions that drive SMN production, such as gene therapy, transcriptional promoters, antisense oligonucleotides, and small molecules, have been demonstrated to mitigate the spinal muscular atrophy (SMA) disease phenotype. Similarly, addressing the aberrant SMN degradation mechanisms, ubiquitination and autophagy, has also been shown to improve neuromuscular health.

For example, the phosphorylated form of E26 transformation-specific domain-containing protein (ELK) inhibits transcription of SMN2, while the binding of cAMP response element-binding protein (CREB) and signal transducer and activator of transcription 5 (STAT5) stimulate the transcription of SMN2[28].

Recent work from Frédéric Charbonnier's lab has demonstrated that ELK and CREB activity are regulated via mitogen-activated protein kinase (MAPK) signaling pathways. SMA mice exhibited a suppression of the Ca^{2+}/calmodulin-dependent kinase II (CaMKII)/phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT)/CREB pathway and activation of MAPK/extracellular regulated kinase (ERK)/ELK cascade which, on balance, inhibited the transcription of the SMN2 gene[29]. Moreover, the stimulation of intracellular CaMKII/PI3K/AKT/CREB signaling was attributed to the upregulation of SMN protein in spinal cord cultures from the Taiwanese SMA type 2 animals and the more severe SMA mice. Of note, the intracellular crosstalk between ERK and AKT pathways further regulate the expression of SMN. Inhibition of ERK/ELK pathway, on balance, increases SMN2 gene expression[29]. These findings were verified by Biondi and colleagues, who have demonstrated that increasing circulating insulin-like growth factor-1 (IGF-1), via transgenic knockout of the IGF-1 receptor, results in the alterations of these pathways[28].

The Janus kinase (JAK)/STAT signaling pathway also regulates SMN2 expression. The activation of JAK tyrosine kinases phosphorylates a group of downstream STAT transcription factors. The administration of prolactin, a peptide hormone known to elicit the JAK2/STAT5 pathway, results in an increase in SMN2 mRNA expression and protein in neuronal cells. Furthermore, in vivo administration of prolactin improves disease phenotype and elongates the lifespan of severe type 1 SMA mice[30].

ii. Post-transcriptional regulation

Post-transcriptional modifications occur to improve the stability and/or the function of mRNA, thus providing an additional layer of gene expression regulation. Immediately after gene transcription, the primary RNA transcript undergoes RNA-splicing events, involving the removal of introns and ligation of exons. Through this process, a single gene may form multiple distinct mRNAs that may result in alternative translation products. Splicing is regulated by the macromolecular structure known as the spliceosome, which is comprised of several snRNPs. The recognition of conventional splice sites and removal of introns are mediated by these snRNPs. Gene editing events are able to inhibit or promote the enhancing or silencing regions in pre-mRNA.

Indeed, the severity of SMA is dictated by the amount of cellular SMN protein and can be mitigated by promoting the transcription of the SMN2 gene, as mentioned above. Splicing mechanisms, which enhance the expression of full-length SMN mRNA, benefit individuals affected by SMA. In fact, antisense oligonucleotides, available to SMA patients, redirect alternative splicing patterns to mitigate the disease phenotype[31–33]. Thus, understanding mechanisms that regulate mRNA splicing and processing is important when discussing SMN regulation. Although the single base difference between SMN1 and SMN2 does not change the amino acid coding, the splicing pattern of the transcript product is altered. The C to T substitution that occurs in the SMN2 gene generates an exonic splicing silencer where the splicing suppressing factor, heterogeneous nuclear ribonucleoprotein (hnRNP) A1, can bind. In addition, the point mutation also disrupts an exonic splicing enhancer, where the splicing activator ASF/SF2 binds. Collectively, these two events occur simultaneously, regulating the splicing of SMN transcript.

An addition, gene expression regulation is through mRNA processing by RNA-binding proteins (RBP). mRNA degradation pathways are initiated by the degradation of

the 3' poly-A tail or the of the 5' mm7GppN cap structure. The remaining mRNA undergoes exonuclease-mediated rapid degradation. Exonucleolytic cleavage of the mRNA is driven by exosomes, a large multi-protein complex, that bind to the adenylate- and uridylate (AU)-rich elements (ARE) located in the 3' untranslated region of targeted mRNA[34]. The ubiquitously expressed RBP, K homology-type splicing regulatory protein (KSRP) can bind to the AREs of mRNA to facilitate the recruitment of exosomes and encourage degradation[35]. Some RBP can prevent ARE-dependent mRNA degradation by competitively binding to the ARE, thus improving the stability of the mRNA. For example, RBPs that are part of the Hu family have a high affinity for AREs, and the overexpression of HuR can increase the half-life of short-lived mRNA[34].

As mentioned above, in all SMA patients, 80-90% of the transcripts produced from SMN2 are truncated, while the remaining 10-20% are full length and can be translated to functional SMN protein. Although the stability of truncated SMN transcript (SMNΔ7) is volatile, some of its function is preserved. Thus, improving the stability and enhancing the expression of this non-toxic mRNA is likely beneficial for individuals affected by SMA. Farooq and colleagues demonstrate the therapeutic efficacy of mRNA stabilization via a series of elaborate experiments. The pharmacological activation of p38 MAPK in severe SMA mice increased SMN protein expression and improved survival in a HuR-dependent manner[36,37]. Specifically, the phosphorylation of p38 MAPK encouraged the translocation of nuclear HuR to the cytosol, where it binds to the ARE of SMN mRNA and augments its stability[36]. Moreover, preventing the decapping of the SMN mRNA has also been demonstrated to prolong lifespan, preserve motor function, and prevent muscle atrophy in SMA mice[38,39]. The oral administration of RG3039, an inhibitor of an RNA decapping

enzyme known as DcpS, increases the expression of full-length and truncated SMN mRNA transcript.

iii. Degradation of SMN

SMN expression is also regulated at the level of protein degradation. SMN protein can be degraded through the ubiquitin-proteasome system and macroautophagy processes[40–42]. Ubiquitination of SMN allows the cargo docking protein, p62/SQSTM1 to bind, thus flagging the targeted protein for degradation. Interestingly, when the autophagy-mediated degradation of these proteins is prevented, the survival of SMA mice improves, which further supports that the regulation of SMN at the degradation level is important. Recent work has also demonstrated heightened autophagosomal degradation in the skeletal muscles of *Smn*$^{2B/-}$ mice[20]. Specifically, these symptomatic SMA mice exhibited a higher expression of the insoluble autophagosome-bound Microtubule-associated protein 1A/1B-light chain 3 (LC3) protein, greater autophagy-related gene expression, and presence of autophagic vacuoles.

5. Treatments to address SMA

Until recently, therapies have exclusively addressed the symptoms of SMA in all of its forms. As of December 2016, the United States Food and Drug Administration approved the antisense oligonucleotide (ASO) Nusinersen, marketed as Spinraza, for use in pediatric and adult SMA patients marking a new era for SMA therapeutics. The availability of this drug highlights efforts to investigate alternative therapies that work through SMN-dependent and -independent mechanisms, which may be used in combination with this groundbreaking drug. Hence, this section will be devoted to reviewing SMN-dependent and -independent therapeutic approaches.

i. SMN-dependent therapies

Increasing the production of SMN from SMN2 proves to be a viable and attractive therapy for SMA as all patients will harbor a copy of the SMN2 gene. As mentioned above, one of the sites for SMN gene regulation is modifying the rate of transcription. Histone deacetylases (HDACs) are expressed in all cell types and normally suppress active gene expression. Pharmacological inhibition of HDACs can enhance SMN expression in patient-derived cells and SMA mouse models, however, the efficacy of these compounds had minimal or no effect for type 2 or 3 SMA patients[43–45]. d'Ydewalle and colleagues have recently identified an endogenous long non-coding RNA that rises from the anti-sense strand of SMN, SMN-AS1, which transcriptionally represses SMN expression[46]. The targeted decay of SMN-AS1 induces greater SMN expression in αMNs of SMA mice. The administration of SMN-AS1 in conjunction with splice switching ASOs results in a cumulative expression of SMN.

Modulation of post-transcriptional mechanisms is also an attractive therapy. Perhaps the most promising and accepted therapy is the use of gene editing molecules, which alter mRNA splicing by interfering with crucial splicing factors. Spinraza, a first generation ASO, displaces the splicing suppressing factor, hnRNP, from the intronic splicing suppressor, encouraging the conventional splicing of SMN2 mRNA[47]. Administration of Spinraza selectively improves motor function in type 1 SMA patients. Of note, this finding was likely due to the variability between disease manifestation and time of drug treatment between patients[48].

In addition, gene replacement is an emerging and promising therapy that also restores SMN expression. The single intravenous injection of the self-complementary adeno-associated virus (AAV) serotype 9, AVXS-101, has been shown to replenish SMN in the spinal cords of SMA mice[47,49–51]. This intervention has recently completed a clinical

safety and efficacy study in type 1 SMA patients. The absence of adverse events suggests that the treatment was safe and tolerable in patients, while improvements of motor function were also observed[52].

ii. SMN-independent therapies

Although the approval of Spinraza has the potential to be revolutionary for SMA patients, the high cost represents a practical barrier for many patients and their families. During the first year of treatment, the cost of Spinraza can cost as much as $750,000 USD, while the annual cost for subsequent years of treatment is halved. Furthermore, biological limitations of the compound may include its sole dependence on SMN-mediated correction of the SMA pathology, as well as its distribution and effectiveness in peripheral tissues such as skeletal muscle. Indeed, recent studies have demonstrated that the periphery must be targeted in order to elicit maximum benefits from current generation ASOs[53,54]. Nonetheless, with the availability and utilization of Spinraza, patients will exhibit less severe disease phenotypes, creating a demand to address the secondary multisystem complications of the disorder. As such, researchers have highlighted a priority for identifying SMN-independent therapies to further mitigate the SMA pathology.

One particular neuroprotectant that was recently part of stage 3 clinical trials is Olesoxime. The drug aims to prevent mitochondrial permeability transition under stress conditions[55]. During phase 2 clinical trials, a study revealed that nonambulatory SMA patients exhibited modest functional improvements with minimal adverse events. Olesoxime has now been discontinued for SMA patients but remains to be a possible therapy for amyotrophic lateral sclerosis patients. Another emerging SMN-independent therapy is drugs that preserve muscle function and reduces fatigue. CK-2127107 and SRK-015 are examples of these molecules and are currently being tested in SMA patients[56,57].

Beyond these SMN-independent therapies that are in the advanced stages of development, the discoveries of other potential therapeutic pathways are ongoing. These therapies address dysfunctional cellular pathways that are evident in the SMA pathology. For example, the pharmacologic inhibition of the ROCK/RhoA signaling cascade improves the lifespan and motor function of murine SMA models by correcting dysregulated actin dynamics[58–60]. Elevated PLS3 expression resulted in prolonged survival of these mice in conjunction with a restoration of actin dynamics and synaptic vesicle transmission[61]. Ubiquitin-like modifier activating enzyme (UBA1), a crucial modifier of ubiquitination, has been shown to be a valid therapeutic target. The application of an AAV-UBA1 has been shown to replenish levels of UBA1 in SMA murine models, resulting in a prolonged lifespan and enhanced motor function[62]. Collectively, these therapies may further mitigate the manifestation of SMA when used alone, or in conjunction with first generation ASOs such as Spinraza. These discoveries have highlighted the importance of identifying SMN-independent pathways and warrant further research to investigate the clinical application of these therapies.

6. Therapeutic application of exercise training

In recent years, the health benefits of exercise have received considerable attention for the treatment of chronic diseases including cardiovascular diseases and metabolic diseases such as obesity and type 2 diabetes[63,64]. In fact, exercise training in combination with dietary interventions has been shown to be more beneficial than some pharmacological therapies[65,66]. Although physical activity elicits multisystem benefits, which have been thoroughly described elsewhere[67–72], the current section will review training adaptations specific to the neuromuscular system. In addition, the following

portion of this section will elaborate on the cellular and molecular mechanisms underlying these training-induced adaptations (Figure 2).

i) Training adaptations in healthy populations

The activity of motor units is essential for locomotion and physical activity. Chronic utilization of αMNs and muscle elicit phenotypic adaptations, which enhance the efficiency of cellular processes within the neuromuscular system. For example, regulatory and transport mechanisms are accelerated in response to exercise training. Fast axonal transport is an essential process that delivers mitochondria and neurotropic-filled vesicles in the anterograde and retrograde direction by kinesin and dynein proteins, respectively[73]. Following a 10-12 week endurance exercise program, the velocity of axonal transport increased in rats[74,75]. In addition, exercise adaptions local to the synapse have been reported. For example, a greater occurrence of nerve terminal branching and neuromuscular hypertrophy is seen in trained rats in an intensity-dependent manner[76,77]. Along these lines, exercise training resulted in increased levels of the vesicle docking protein, synaptosomal-associated protein 25 (SNAP25)[78]. Notably, the increased activity of αMN use has also been demonstrated to also improve synaptic transmission[79–81]. These findings are complemented with electrophysiology experiments that demonstrate improvements in neuron activation. Such effects include a hyperpolarized resting membrane potential, faster rate of hyperpolarization, and greater afterhyperpolarization

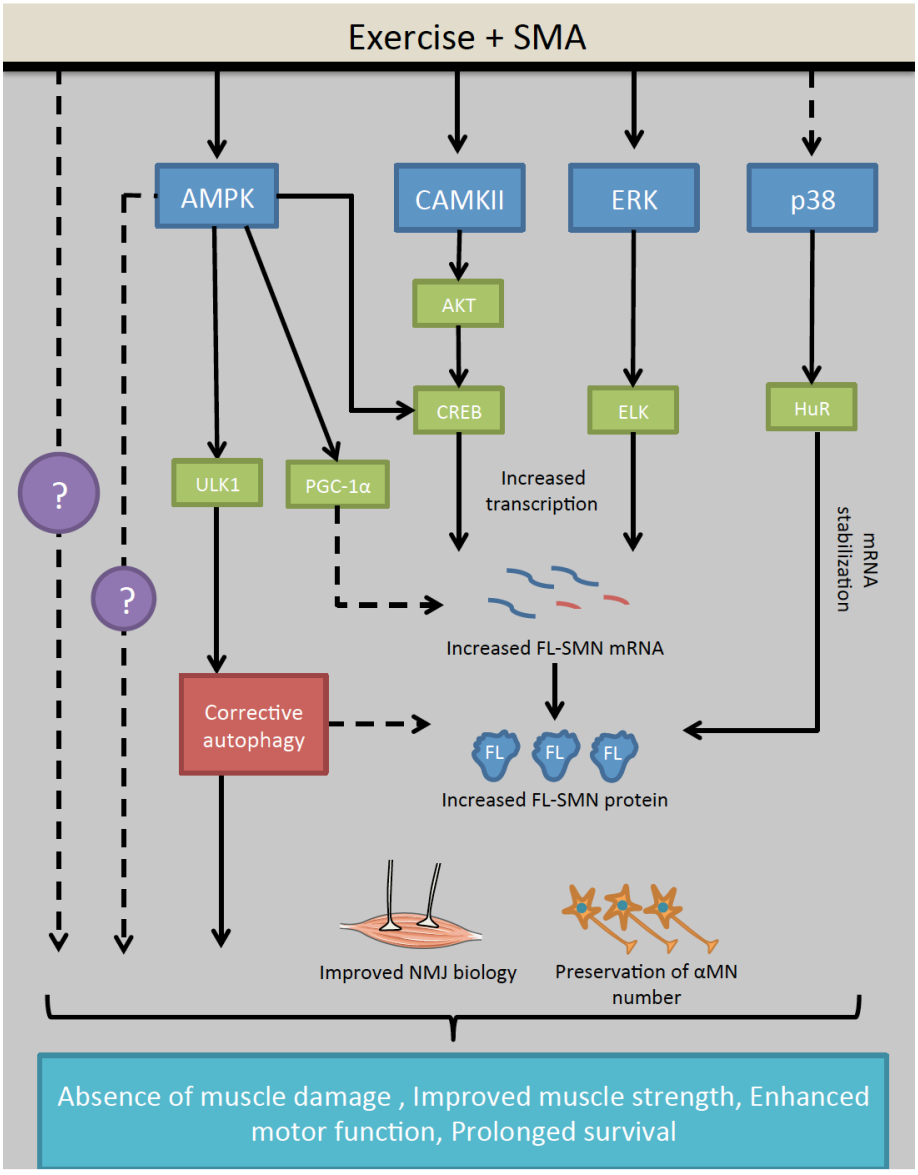

Figure 2. Putative mechanisms of exercise-induced neuromuscular plasticity in spinal muscular atrophy. Studies of physical activity or small molecules in SMA mice demonstrate possible pathways of exercise adaptation. Activation of upstream molecules, for example, adenosine monophosphate-activated protein kinase (AMPK), Ca^{2+}/calmodulin-dependent kinase (CaMKII), and p38 Mitogen-activated protein kinase (p38 MAPK) may initiate a signaling cascade that results in an increase in FL-SMN expression in the neuromuscular system. Exercise-evoked alterations in skeletal muscle and α-motoneurons (αMN) might occur in both SMN-dependent and SMN-independent fashion. These beneficial adaptations result in enhanced healthspan and lifespan of mice with SMA. Dashed lines refer to potential linkages between steps. Question marks indicate alternative, undiscovered pathways and/or mechanisms. Arrows illustrate activation. The blocked line indicates inhibition.

amplitude[82]. Taken together, the increased complexity of the NMJ likely is associated with the improved neural transmission that concurs and suggests that exercise can mediate adaptations at the level of the synapse.

On the distal end of the αMN, the innervated skeletal muscle resides. Physical activity can evoke metabolic, morphologic, and functional changes to this dynamic tissue. For example, resistance exercise training can induce muscle hypertrophy, increase glycolytic capacity, and improve strength, while endurance-type exercise training improves mitochondrial density, oxidative function, and insulin sensitivity of human skeletal muscle[67]. These adaptations collectively contribute to the muscle phenotype, resulting in the adoption of different muscle fiber type characteristics. Aerobic exercise training has been shown to elicit a slower and more oxidative muscle phenotype[83]. Interestingly, factors intrinsic to muscle have a retrograde influence on the αMN[84–86]. The pharmacological induction of a slower and more oxidative muscle phenotype is able to provoke a phenotypic shift in the innervating αMN.

ii) Exercise signaling in healthy populations

A single bout of exercise produces a series of mechanical, biochemical and thermal stimuli, which are interpreted by intracellular signaling cascades and result in a transient bursts of mRNA[63]. Repeated bouts of physical activity result in the accumulation of mRNA burst and drive transcriptional processes which are necessary for training adaptations[87,88]. Specifically, these signaling pathways are evoked by contractile-evoked byproducts, including, but not limited to, sarcoplasmic calcium (Ca^{2+}) cycling, increased adenosine monophosphate (AMP), adenosine diphosphate (ADP), or reduced creatine phosphate concentrations, elevated reactive oxygen species levels, and altered redox states[89]. Although there are many exercise-induced intracellular signaling cascades that have been

previously reviewed[63,90–92], this section will focus on a select few that are relevant in the context of SMA.

AMP-activated protein kinase (AMPK), an important molecule known to regulate catabolic processes, is activated with increased AMP/ATP or ADP/ATP ratios. As physical activity utilizes ATP, AMP and ADP levels increase, thus activating AMPK[93]. Exercise-induced or pharmacological activation of AMPK results in the post-translational modification of transcription factors and in some cases RNA binding proteins. Chronic activation of AMPK alters the metabolic gene profile and induces mitochondrial biogenesis via activation of transcription factors such as nuclear respiratory factor 1, myocyte enhancer factor 2 (MEF2), and HDACs[94]. Furthermore, the exercise-induced activation of AMPK is capable of phosphorylating unc51-like kinase 1 (ULK1), which initiates autophagy-mediated degradation. Autophagy is essential for training-induced skeletal muscle adaptations. Specifically, the heterozygous disruption of beclin-1 diminishes exercise adaptations in skeletal muscle, demonstrating that autophagy is essential for training adaptations to occur[95].

The chemical and mechanical processes activated by muscle contraction also drive the activity of MAPKs. The level of skeletal muscle MAPK activation is partly dependent on the type of exercise, as well as training status[96]. MAPK activation regulates transcriptional, in addition to post-transcriptional, events through phosphorylation of various substrates. For example, exercise-induced activation of p38 MAPK can drive upstream regulators of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), such as activating transcription factor 2 and MEF2[97]. Furthermore, the pharmacological activation of p38 MAPK has been shown to stabilize mRNA via human-

antigen R (HuR)- and K-homology splicing regulatory protein (KSRP)-dependent mechanisms[37,98,99].

Ca^{2+} is essential for facilitating actin-myosin cross bridge interactions during myofibrillar contractions. The cyclical elevations in sarcoplasmic [Ca^{2+}] simulate the CaMK pathway. Contraction-induced alterations in [Ca^{2+}] are essential for mediating phenotypic change in skeletal muscle[100]. For instance, the activation of CaMKII targets various transcription factors such as CREB, MEF2, and HDACs that are important for the regulation of skeletal muscle gene expression[101].

PGC-1α is a transcriptional co-activator known to induce mitochondrial biogenesis, angiogenesis, shift myosin heavy chain isoforms, alter NMJ gene expression, and regulate energy metabolism[102]. Upon activation, this molecule translocates to the nucleus where it binds to transcription factors that regulate an array of genes related to bioenergetics, NMJ maintenance, and inflammation signaling. In addition, PGC-1α-meditated mRNA splicing may also posttranscriptionally alter gene expression[103,104]. Many signaling kinases, such as CaMKII, p38 MAPK, and AMPK converge on this transcriptional co-activator making it a sensitive and potent regulator of metabolism. Transgenic upregulation of this molecule elicits a drastic phenotype shift towards a slower and more oxidative neuromuscular phenotype[105,106].

7. Exercise training in SMA

In addition to the therapeutic application of physical activity in various chronic diseases, exercise training is an emerging therapy for neuromuscular disorders[107,108]. Feasibility and safety of exercise training in this population is dependent on the severity of the disease. Although exercise training is currently not practical for type 1 SMA patients, physical activity poses to be beneficial for patients who are capable (i.e., type 2, 3, and 4

SMA). Furthermore, with the broad approval of Spinraza, the health- and lifespan of SMA patients will likely elongate, creating a broader therapeutic window for these individuals. For these reasons, the following section will highlight literature discussing exercise-training adaptations in SMA patients and preclinical models. Notably, we recently published an invited review on the exercise biology of NMDs, including SMA[108].

i) Training adaptations in SMA patients

McCartney and colleagues at McMaster University were the first to investigate the effects of physical activity in SMA patients[109]. Here, three SMA patients were subjected to a nine-week progressive resistance-training program, three-times/week. Trained upper and lower limbs demonstrated improvements in dynamic strength and greater isokinetic torque generation. Metrics of feasibility, such as the absence of muscular damage, confirmed the tolerability and safety of exercise in this population, despite that SMA patients had been advised against exercising training at the time.

Since then, numerous exercise studies have been conducted in SMA patients, including both resistance- and endurance-type exercises (Table 1). Lewelt and colleagues (2015) investigated the effects of a twelve-week, resistance-training program in type 2 and 3 SMA patients. In addition to the modest, but significant, improvements in strength and motor function, the feasibility and safety of exercise training was further confirmed with this study[110]. Chronic endurance-type exercise has also been demonstrated to benefit SMA patients. A twelve-week, home-based aerobic training program, conducted on type 2 SMA patients, improved fitness (i.e., ~30% increase in VO_{2max}) without the presence of muscle damage[111]. In a separate study, type 2 SMA patients demonstrated exercise-induced improvements in endurance capacity following a twelve-week training study[112]. Taken

together, these studies demonstrate the efficacy and feasibility of exercise training in SMA patients.

ii) Exercise signaling in SMA models

The investigation of molecular mechanisms that underlie exercise-induced neuromuscular remodeling in SMA is important because it increases our understanding of the basic biology of the disease, as well as facilitates the discovery of novel targets for future therapies. To date, there are no studies that have pursued this cause in SMA patients. However, Frédéric Charbonnier and colleagues have addressed this gap via a series of pre-clinical studies which elaborate on the mechanisms of exercise adaptation in the neuromuscular system of Taiwanese SMA mice[28,113–115]. A motorized running wheel program, five-times/week, improved clinical outcomes, including a ~60% prolonged survival rate, diminished muscle weakness, as well as enhanced motor behavior, in SMA animals relative to their sedentary counterparts[28,114–116]. Amongst these adaptations, greater full-length SMN mRNA was present in the lumbar spinal cord and skeletal muscle[28,115]. This rise in SMN mRNA can be explained, in part, by the enhanced CaMKII-protein kinase B (AKT)-CREB signaling axis, which was mediated by the reduction in insulin-like growth factor-1 receptor (IGF-1R) content. Exercise-evoked downregulation of IGF-1R would likely attenuate ERK and ELK activities, which serve to repress full-length SMN induction from SMN2[28]. Trained SMA animals also exhibit attenuated muscle atrophy and a reduced loss of αMNs[115]. Along these lines, exercise positively influences NMJ morphology and transmission efficiency. The authors also observed a heightened expression of a subunit of the N-methyl-D-aspartate receptor (NMDAR), GluR epsilon 1 (NR2A), and when inhibited, the observed training adaptations where mitigated in αMNs and skeletal muscle[117].

Table 1: Summary of exercise studies with SMA patients

Exercise mode	*Participant cohort size*	*SMA type*	*Exercise protocol*	*Safety*	*Adherence*	*Effects*	*Reference*
Arm cycle ergometer	7	Type 2	12 week training program; 3x per week; 30 minute sessions at 60% MHR	Exercise training was well tolerated; no reports of myalgia	Two participants dropped out	Increase in cycling distance and duration at weeks 6 and 12 compared to pre-exercise values; no change in peripheral blood SMN expression	Bora et al., 2018
Leg cycle ergometer	8	Type 3	12 week training program; 2-4x per week; 30 minute sessions at 60-75% VO_{2max}	No training-induced increases in plasma creatine kinase	Two participants dropped out	27% increase in VO_{2max}; 3 patients reported improved muscle strength, 2 reported increased levels of activity, 6 reported increased fatigue	Madsen et al., 2015
Leg cycle ergometer; resistance exercise	14	Type 3	6 month training program; 5x per week; whole body resistance training, 3x per week; 30 minute sessions; 60-80% 1RM	Exercise was well tolerated; no adverse events reported	12 participants completed the exercise training protocol	~5% increase in VO_{2max}; no change in MMT; no change in 6MWT or clinical measures of motor function	Montes et al., 2015
Resistance exercise	9	Type 2 and type 3	12 week training program; 3x per week; progressive resistance training	Training sessions were 99.5% pain-free; no adverse events reported	High participant adherence (90.4%)	Increased muscle strength revealed by MMT; improvement in MHFMSE score	Lewelt et al., 2015
Resistance exercise	3	Not stated - likely to be mild SMA	9 week training program; 3x per week; progressive resistance training from 40-70% MVC; single arm and bilateral leg	Muscle biopsy and computerized tomography revealed no training-induced muscle damage	All participants completed training	Improved dynamic strength in the arms and legs; greater isokinetic torque generation, and enhanced elbow flexor contractile properties	McCartney et al., 1988
Resistance exercise	3	Not stated - likely to be type 3 or 4	12 month training program; 4x per week; whole body resistance training	Not stated	Participants did not experience overwork weakness.	Elbow flexion was not reported due to the lack of initial strength; maximal knee extension force increased ~100%	Milner-Brown and Miller 1988

6MWT = 6 minute walk test; MHFMSE = Modified Hammersmith Functional Motor Scale-Extend; MHR = maximal heart rate; MMT = Manual Muscle Testing; MVC = maximal voluntary contraction; RM = repetition max; SMA = spinal muscular atrophy; SMN = survival motor neuron.

AMPK and p38 MAPK may also contribute to exercise adaptations in SMA[106,108]. As mentioned above, it is well established that endurance-type exercise training benefits occur due to, in part, the chronic activation of these kinases[63,67]. Chronic pharmacological activation of AMPK protects NMJ morphology, as well as elicits an increase in myofiber size and proportions of type I fibres in severe SMA mice, while treatment with the p38 MAPK activator celecoxib induces SMN mRNA stabilization by a HuR-dependent manner in the brain and spinal cord of these animals[118,119]. Activation of p38 MAPK has also been demonstrated to stabilize mRNA through a KSRP-dependent mechanism, which is impaired by the absence of SMN[120]. Based on these studies, it is, therefore, reasonable to hypothesize that AMPK- and p38 MAPK-mediated signaling are among the mechanisms responsible for driving exercise adaptation in SMA.

8. Study Objectives

Recent investigations of the utility of exercise training as a therapeutic modality for SMA demonstrate that physical activity is safe and can elicit some physiological benefits[108]. However, the cellular and molecular mechanisms of these training-induced adaptations have yet to be fully determined. Hence, expanding our knowledge of the cellular events stimulated by an acute bout of exercise in a model of SMA may reveal novel pathways for further therapeutic investigation. Thus, the purpose of this thesis is two-fold. First, using an established pre-clinical murine model of type 2/3 SMA at pre- and post-symptomatic timepoints along disease progression, we wish to investigate the expression and activation of molecules that are important for maintaining and remodeling a robust neuromuscular phenotype. Second, we seek to elucidate the intracellular signaling cascades that are simulated by a single episode of endurance-type exercise, and whether these pathways are associated with the induction of SMN gene expression. We focus our studies

on the skeletal muscle of SMA animals due to the emerging consensus among SMA experts that factors intrinsic to muscle play an important role in pathogenesis[3]. Furthermore, its plastic attributes[67,89], as well as its abundance and accessibility, makes skeletal muscle an attractive tissue to study. We hypothesize that 1) the expression and function of molecules that maintain and remodel muscle phenotype, such as AMPK, p38 MAPK, and PGC-1α, will be altered during disease progression in the type 2/3 SMA-like *Smn*$^{2B/-}$ mouse model compared to their healthy littermate controls, and 2) a single bout of exercise will evoke favourable changes in intracellular signaling and gene expression, including those processes germane to SMN, in the skeletal muscle of SMA-like animals.

9. References

1. Awano, T., Kim, J.-K. & Monani, U. R. Spinal Muscular Atrophy: Journeying From Bench to Bedside. *Neurotherapeutics* 786–795 (2014). doi:10.1007/s13311-014-0293-y
2. Armstrong, E. P. *et al.* The economic burden of spinal muscular atrophy. *J. Med. Econ.* **19,** 822–826 (2016).
3. Hamilton, G. & Gillingwater, T. H. Spinal muscular atrophy: Going beyond the motor neuron. *Trends in Molecular Medicine* **19,** 40–50 (2013).
4. Rudnik-Schöneborn, S. *et al.* Congenital heart disease is a feature of severe infantile spinal muscular atrophy. *J. Med. Genet.* **45,** 635–638 (2008).
5. Prufer De Queiroz, A., Araujo, C., Araujo, M., Swoboda, K. J. & Prufer Queiroz, A. Vascular Perfusion Abnormalities in Infants with Spinal Muscular Atrophy. *J Pediatr* **155,** 292–294 (2009).
6. Rudnik-Schöneborn, S. *et al.* Digital necroses and vascular thrombosis in severe spinal muscular atrophy. *Muscle Nerve* **42,** 144–147 (2010).
7. Boyd, P. J. *et al.* Bioenergetic status modulates motor neuron vulnerability and pathogenesis in a zebrafish model of spinal muscular atrophy. *PLoS Genet.* **13,** (2017).
8. Lefebvre, S. *et al.* Correlation between severity and SMN protein level in spinal muscular atrophy. *Nat. Genet.* **16,** 265–269 (1997).
9. Hsieh-Li, H. M. *et al.* A mouse model for spinal muscular atrophy. *Nat. Genet.* **24,** 66–70 (2000).
10. Monani, U. R. *et al.* The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in Smn(-/-) mice and results in a mouse with spinal

muscular atrophy. *Hum. Mol. Genet.* **9,** 333–339 (2000).

11. Bowerman, M., Murray, L. M., Beauvais, A., Pinheiro, B. & Kothary, R. A critical smn threshold in mice dictates onset of an intermediate spinal muscular atrophy phenotype associated with a distinct neuromuscular junction pathology. *Neuromuscul. Disord.* **22,** 263–276 (2012).
12. Cooper, T. A., Wan, L. & Dreyfuss, G. RNA and Disease. *Cell* **136,** 777–793 (2009).
13. Will, C. L. & Lührmann, R. Spliceosomal UsnRNP biogenesis, structure and function. *Curr. Opin. Cell Biol.* **13,** 290–301 (2001).
14. Wan, L. *et al.* The Survival of Motor Neurons Protein Determines the Capacity for snRNP Assembly : Biochemical Deficiency in Spinal Muscular Atrophy The Survival of Motor Neurons Protein Determines the Capacity for snRNP Assembly : Biochemical Deficiency in Spinal Muscul. *Mol. Cell. Biol.* **25,** 5543–5551 (2005).
15. Bowerman, M. *et al.* Therapeutic strategies for spinal muscular atrophy: SMN and beyond. *Dis. Model. Mech.* **10,** 943–954 (2017).
16. Hensel, N. & Claus, P. The Actin Cytoskeleton in SMA and ALS: How Does It Contribute to Motoneuron Degeneration? *Neuroscientist* (2018). doi:10.1177/1073858417705059
17. Donlin-Asp, P. G., Bassell, G. J. & Rossoll, W. A role for the survival of motor neuron protein in mRNP assembly and transport. *Curr. Opin. Neurobiol.* **39,** 53–61 (2016).
18. Donlin-Asp, P. G. *et al.* The Survival of Motor Neuron Protein Acts as a Molecular Chaperone for mRNP Assembly. *Cell Rep.* **18,** (2017).
19. Groen, E. J. N. Temporal and tissue-specific variability of SMN protein levels in mouse models of spinal muscular atrophy. *Eur. J. Paediatr. Neurol.* **21,** e13 (2017).

20. Deguise, M.-O. *et al.* Differential induction of muscle atrophy pathways in two mouse models of spinal muscular atrophy. *Sci. Rep.* **6,** 28846 (2016).

21. Boyer, J. G. *et al.* Early onset muscle weakness and disruption of muscle proteins in mouse models of spinal muscular atrophy. *Skelet. Muscle* **3,** 24 (2013).

22. Boyer, J. G. *et al.* Myogenic program dysregulation is contributory to disease pathogenesis in spinal muscular atrophy. *Hum. Mol. Genet.* **23,** 4249–4259 (2014).

23. Kariya, S. *et al.* Requirement of enhanced Survival Motoneuron protein imposed during neuromuscular junction maturation. *J. Clin. Invest.* **124,** 785–800 (2014).

24. Kariya, S. *et al.* Reduced SMN protein impairs maturation of the neuromuscular junctions in mouse models of spinal muscular atrophy. *Hum. Mol. Genet.* **17,** 2552–2569 (2008).

25. Deguise, M. O. *et al.* Immune dysregulation may contribute to disease pathogenesis in spinal muscular atrophy mice. *Hum. Mol. Genet.* **26,** 801–819 (2017).

26. Groen, E. J. N., Talbot, K. & Gillingwater, T. H. Advances in therapy for spinal muscular atrophy: promises and challenges. *Nat. Rev. Neurol.* (2018). doi:10.1038/nrneurol.2018.4

27. Boda, B. *et al.* Survival motor neuron SMN1 and SMN2 gene promoters: identical sequences and differential expression in neurons and non-neuronal cells. *Eur. J. Hum. Genet.* **12,** 729–737 (2004).

28. Biondi, O. *et al.* IGF-1R Reduction Triggers Neuroprotective Signaling Pathways in Spinal Muscular Atrophy Mice. *J. Neurosci.* **35,** 12063–12079 (2015).

29. Branchu, J. *et al.* Shift from Extracellular Signal-Regulated Kinase to AKT/cAMP Response Element-Binding Protein Pathway Increases Survival-Motor-Neuron Expression in Spinal-Muscular-Atrophy-Like Mice and Patient Cells. *J. Neurosci.*

33, 4280–4294 (2013).

30. Farooq, F. *et al.* Prolactin increases SMN expression and survival in a mouse model of severe spinal muscular atrophy via the STAT5 pathway. *J Clin Invest* **121,** (2011).
31. Hua, Y., Vickers, T. A., Okunola, H. L., Bennett, C. F. & Krainer, A. R. Antisense Masking of an hnRNP A1/A2 Intronic Splicing Silencer Corrects SMN2 Splicing in Transgenic Mice. *Am. J. Hum. Genet.* **82,** 834–848 (2008).
32. Chiriboga, C. A. *et al.* Results from a phase 1 study of nusinersen (ISIS-SMNRx) in children with spinal muscular atrophy. *Neurology* **86,** 890–7 (2016).
33. Finkel, R. S. *et al.* Treatment of infantile-onset spinal muscular atrophy with nusinersen: a phase 2, open-label, dose-escalation study. *Lancet* **388,** 3017–3026 (2016).
34. Eberhardt, W., Doller, A., Akool, E. S. & Pfeilschifter, J. Modulation of mRNA stability as a novel therapeutic approach. *Pharmacology and Therapeutics* **114,** 56–73 (2007).
35. Briata, P., Chen, C.-Y., Ramos, A. & Gherzi, R. Functional and molecular insights into KSRP function in mRNA decay ☆. *BBA - Gene Regul. Mech.* **1829,** 689–694 (2013).
36. Farooq, F., Balabanian, S., Liu, X., Holcik, M. & MacKenzie, A. p38 Mitogen-activated protein kinase stabilizes SMN mRNA through RNA binding protein HuR. *Hum. Mol. Genet.* **18,** 4035–4045 (2009).
37. Farooq, F. *et al.* Celecoxib increases SMN and survival in a severe spinal muscular atrophy mouse model via p38 pathway activation. *Hum. Mol. Genet.* **22,** 3415–3424 (2013).
38. Van Meerbeke, J. P. *et al.* The DcpS inhibitor RG3039 improves motor functionin

SMA mice. *Hum. Mol. Genet.* **22,** 4074–4083 (2013).

39. Gogliotti, R. G. *et al.* The dcpS inhibitor RG3039 improves survival, function and motor unit pathologies in two SMA mouse models. *Hum. Mol. Genet.* **22,** 4084–4101 (2013).

40. Kwon, D. Y., Motley, W. W., Fischbeck, K. H. & Burnett, B. G. Increasing expression and decreasing degradation of SMN ameliorate the spinal muscular atrophy phenotype in mice. *Hum. Mol. Genet.* **20,** 3667–3677 (2011).

41. Kwon, D. Y. *et al.* The E3 ubiquitin ligase mind bomb 1 ubiquitinates and promotes the degradation of survival of motor neuron protein. *Mol. Biol. Cell* **24,** 1863–1871 (2013).

42. Rodriguez-Muela, N. *et al.* Blocking p62/SQSTM1-dependent SMN degradation ameliorates Spinal Muscular Atrophy disease phenotypes. *J. Clin. Invest.* (2018). doi:10.1172/JCI95231

43. Mercuri, E. *et al.* Towards harmonisation of outcome measures for DMD and SMA within TREAT-NMD; Report of three expert workshops: TREAT-NMD/ENMC Workshop on outcome measures, 12th–13th May 2007, Naarden, The Netherlands; TREAT-NMD Workshop on outcome measures in experimental trials for DMD, 30th June–1st July 2007, Naarden, The Netherlands; Conjoint Institute of Myology TREAT-NMD Meeting on physical activity monitoring in neuromuscular disorders, 11th July 2007, Paris, France. 12–13 (2007). doi:10.1016/j.nmd.2008.07.003

44. Swoboda, K. J. *et al.* SMA CARNI-VAL trial part I: Double-blind, randomized, placebo-controlled trial of L-carnitine and valproic acid in spinal muscular atrophy. *PLoS One* **5,** (2010).

45. Kissel, J. T. *et al.* SMA CARNI-VAL TRIAL PART II: A Prospective, Single-

Armed Trial of L-Carnitine and Valproic Acid in Ambulatory Children with Spinal Muscular Atrophy. at <www.plosone.org>

46. d'Ydewalle, C. *et al.* The Antisense Transcript SMN-AS1 Regulates SMN Expression and Is a Novel Therapeutic Target for Spinal Muscular Atrophy. *Neuron* **93,** 66–79 (2017).

47. Passini, M. A. *et al.* Antisense oligonucleotides delivered to the mouse CNS ameliorate symptoms of severe spinal muscular atrophy. *Sci. Transl. Med.* **3,** 72ra18 (2011).

48. Finkel, R. S. *et al.* Nusinersen versus Sham Control in Infantile-Onset Spinal Muscular Atrophy. *N. Engl. J. Med.* **377,** 1723–1732 (2017).

49. Dominguez, E. *et al.* Intravenous scAAV9 delivery of a codon-optimized SMN1 sequence rescues SMA mice. *Hum. Mol. Genet.* **20,** 681–693 (2011).

50. Foust, K. D. *et al.* Rescue of the spinal muscular atrophy phenotype in a mouse model by early postnatal delivery of SMN. *Nat. Biotechnol.* **28,** 271–274 (2010).

51. Valori, C. F. *et al.* Systemic Delivery of scAAV9 Expressing SMN Prolongs Survival in a Model of Spinal Muscular Atrophy. *Sci. Transl. Med.* **2,** 35ra42-35ra42 (2010).

52. Mendell, J. *et al.* AVXS-101 Phase 1 Gene Therapy Clinical Trial in SMA Type 1: Event Free Survival and Achievement of developmental milestones (CT.003). *Neurology* **88,** (2017).

53. Hua, Y. *et al.* Peripheral SMN restoration is essential for long-term rescue of a severe spinal muscular atrophy mouse model. *Nature* **478,** 123–126 (2011).

54. Hua, Y. *et al.* Motor neuron cell-nonautonomous rescue of spinal muscular atrophy phenotypes in mild and severe transgenic mouse models. *Genes Dev.* **29,** 288–97

(2015).

55. Bertini, E. *et al.* Safety and efficacy of olesoxime in patients with type 2 or non-ambulatory type 3 spinal muscular atrophy: a randomised, double-blind, placebo-controlled phase 2 trial. *Lancet Neurol.* **4422,** (2017).

56. Hwee, D. T. *et al.* The Small-Molecule Fast Skeletal Troponin Activator, CK-2127107, Improves Exercise Tolerance in a Rat Model of Heart Failure s. *J. Pharmacol. Exp. Ther. J Pharmacol Exp Ther* **353,** 159–168 (2015).

57. Groen, E. J. N., Talbot, K. & Gillingwater, T. H. Advances in therapy for spinal muscular atrophy: promises and challenges. *Nat. Rev. Neurol.* (2018). doi:10.1038/nrneurol.2018.4

58. Bowerman, M., Beauvais, A., Anderson, C. L. & Kothary, R. Rho-kinase inactivation prolongs survival of an intermediate SMA mouse model. *Hum. Mol. Genet.* **19,** 1468–1478 (2010).

59. Bowerman, M., Shafey, D. & Kothary, R. Smn depletion alters profilin II expression and leads to upregulation of the RhoA/ROCK pathway and defects in neuronal integrity. *J. Mol. Neurosci.* **32,** 120–131 (2007).

60. Bowerman, M., Murray, L. M., Boyer, J. G., Anderson, C. L. & Kothary, R. Fasudil improves survival and promotes skeletal muscle development in a mouse model of spinal muscular atrophy. *BMC Med.* **10,** 24 (2012).

61. Hosseinibarkooie, S. *et al.* The Power of Human Protective Modifiers: PLS3 and CORO1C Unravel Impaired Endocytosis in Spinal Muscular Atrophy and Rescue SMA Phenotype. *Am. J. Hum. Genet.* **99,** 647–665 (2016).

62. Powis, R. A. *et al.* Systemic restoration of UBA1 ameliorates disease in spinal muscular atrophy. *JCI insight* **1,** e87908 (2016).

63. Egan, B. & Zierath, J. R. Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell Metabolism* **17,** 162–184 (2013).

64. Fan, W. & Evans, R. M. Exercise Mimetics: Impact on Health and Performance. *Cell Metabolism* **25,** 242–247 (2017).

65. Knowler, W. C. *et al.* Reduction in the Incidence of Type 2 Diabetes with Lifestyle Intervention or Metformin. *N. Engl. J. Med.* **346,** 393–403 (2002).

66. Borst, S. E. Interventions for sarcopenia and muscle weakness in older people. *Age and Ageing* **33,** 548–555 (2004).

67. Hawley, J. A., Hargreaves, M., Joyner, M. J. & Zierath, J. R. Integrative biology of exercise. *Cell* **159,** 738–749 (2014).

68. Green, D. J. Exercise training as vascular medicine: Direct impacts on the vasculature in humans. *Exerc. Sport Sci. Rev.* **37,** 196–202 (2009).

69. Lavie, C. J. *et al.* Exercise and the cardiovascular system: Clinical science and cardiovascular outcomes. *Circ. Res.* **117,** 207–219 (2015).

70. Guerrieri, D. & Van Praag, H. Exercise-mimetic AICAR transiently benefits brain function. *Oncotarget* **6,** (2015).

71. Van Praag, H., Fleshner, M., Schwartz, M. W. & Mattson, M. P. Symposium Exercise, Energy Intake, Glucose Homeostasis, and the Brain. (2014). doi:10.1523/JNEUROSCI.2814-14.2014

72. Simpson, R. J., Kunz, H., Agha, N. & Graff, R. Exercise and the Regulation of Immune Functions. *Prog. Mol. Biol. Transl. Sci.* **135,** 355–380 (2015).

73. Millecamps, S. & Julien, J.-P. Axonal transport deficits and neurodegenerative diseases. *Nat. Rev. Neurosci.* **14,** 161–176 (2013).

74. Jasmin, B. J., Lavoie, P.-A. & Gardiner, P. F. Fast axonal transport of

acetylcholinesterase in rat sciatic motoneurons is enhanced following prolonged daily running, but not following swimming. *Neurosci. Lett.* **78,** 156–172 (1987).

75. Jasmin, B. J., Lavoie, P., Gardiner, P. F. & GARDINER Fast, P. F. Fast axonal transport of labeled proteins in motoneurons of exercise-trained rats. (1988). at <www.physiology.org/journal/ajpcell>

76. Andonian, M. & Fahim*, M. Endurance Exercise Alters the Morphology of Fast- and Slow-Twitch Rat Neuromuscular Junctions. *Int. J. Sports Med.* **09,** 218–223 (1988).

77. Deschenes, M. R. *et al.* The effects of exercise training of different intensities on neuromuscular junction morphology. *J. Neurocytol.* **22,** 603–615 (1993).

78. Kang, C. M., Lavoie, P. A. & Gardiner, P. F. Chronic exercise increases SNAP-25 abundance in fast-transported proteins of rat motoneurones. *Neuroreport* **6,** 549–553 (1995).

79. Argaw, A., Desaulniers, P. & Gardiner, P. F. ENHANCED NEUROMUSCULAR TRANSMISSION EFFICACY IN OVERLOADED RAT PLANTARIS MUSCLE. *Muscle Nerve* **29,** 97–103 (2004).

80. Desaulniers, P. *et al.* Habitual exercise enhances neuromuscular transmission efficacy of rat soleus muscle in situ Habitual exercise enhances neuromuscular transmission efficacy of rat soleus muscle in situ. 1041–1048 (2014). at <http://www.jap.org>

81. M., D. *et al.* Effects of enhanced activity on synaptic transmission in mouse extensor digitorum longus muscle. *J. Physiol.* **436,** 283–292 (1991).

82. Gardiner, P. F. Changes in alpha-motoneuron properties with altered physical activity levels. *Exerc. Sport Sci. Rev.* **34,** 54–8 (2006).

83. Saltin, B., Henriksson, J., Nygaard, E., Andersen, P. & Jansson, E. FIBER TYPES AND METABOLIC POTENTIALS OF SKELETAL MUSCLES IN SEDENTARY MAN AND ENDURANCE RUNNERS. *Ann. N. Y. Acad. Sci.* **301,** 3–29 (1977).

84. Chakkalakal, J. V, Nishimune, H., Ruas, J. L., Spiegelman, B. M. & Sanes, J. R. Retrograde influence of muscle fibers on their innervation revealed by a novel marker for slow motoneurons. *Development* **137,** 3489–3499 (2010).

85. Arnold, A.-S. *et al.* ARTICLE Morphological and functional remodelling of the neuromuscular junction by skeletal muscle PGC-1a. *Nat. Commun.* **5,** (2014).

86. Mills, R. *et al.* Neurturin is a PGC-1α1-controlled myokine that promotes motor neuron recruitment and neuromuscular junction formation. *Mol. Metab.* **7,** 12–22 (2018).

87. Neufer, P. D. & Dohm, G. L. Exercise induces a transient increase in transcription of the GLUT-4 gene in skeletal muscle. *Am. J. Physiol.* **265,** C1597–C1603 (1993).

88. Perry, C. G. R. *et al.* Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. *J. Physiol.* **588,** 4795–4810 (2010).

89. Hawley, J. A., Hargreaves, M. & Zierath, J. R. Signalling mechanisms in skeletal muscle: role in substrate selection and muscle adaptation. *Essays Biochem.* **42,** 1–12 (2006).

90. Bassel-Duby, R. & Olson, E. N. Signaling Pathways in Skeletal Muscle Remodeling. *Annu. Rev. Biochem.* **75,** 19–37 (2006).

91. Coffey, V. G. & Hawley, J. A. The molecular bases of training adaptation. *Sports Medicine* **37,** 737–763 (2007).

92. Hood, D. A., Irrcher, I., Ljubicic, V. & Joseph, A. M. Coordination of metabolic

plasticity in skeletal muscle. *J. Exp. Biol.* **209,** 2265–2275 (2006).

93. Jäger, S. S., Handschin, C. C., St-Pierre, J. J. & Spiegelman, B. M. B. M. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. *Pnas* **104,** 12017–12022 (2007).

94. Bergeron, R. *et al.* Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. *Am. J. Physiol. Endocrinol. Metab.* **281,** E1340-6 (2001).

95. Lira, V., Okutsu, M., Greene, N. & Laker, R. C. Autophagy is required for exercise training-induced skeletal muscle adaptation and improvement of physical performance. *Artic. FASEB J.* (2013). doi:10.1096/fj.13-228486

96. Yu, M. *et al.* Metabolic and mitogenic signal transduction in human skeletal muscle after intense cycling exercise. *J. Physiol.* **546,** 327–335 (2003).

97. Akimoto, T. *et al.* Exercise stimulates Pgc-1alpha transcription in skeletal muscle through activation of the p38 MAPK pathway. *J. Biol. Chem.* **280,** 19587–19593 (2005).

98. Péladeau, C., Adam, N. J. & Jasmin, B. J. Celecoxib treatment improves muscle function in mdx mice and increases utrophin A expression. *FASEB J.* **0,** fj.201800081R (2018).

99. Péladeau, C. *et al.* Combinatorial therapeutic activation with heparin and AICAR stimulates additive effects on utrophin A expression in dystrophic muscles. *Hum. Mol. Genet.* **25,** 24–43 (2016).

100. Rose, A. J., Kiens, B. & Richter, E. A. Ca 2+ –calmodulin-dependent protein kinase expression and signalling in skeletal muscle during exercise. *J Physiol* **5743,** 889–903 (2006).

101. Liu, Y., Randall, W. R. & Schneider, M. F. Activity-dependent and -independent nuclear fluxes of HDAC4 mediated by different kinases in adult skeletal muscle. *J. Cell Biol.* **168,** 887–97 (2005).

102. Dial, A. G., Ng, S. Y., Manta, A. & Ljubicic, V. The Role of AMPK in Neuromuscular Biology and Disease. *Trends Endocrinol. Metab.* **29,** 300–312 (2018).

103. Martínez-Redondo, V. *et al.* Peroxisome proliferator-activated receptor γ coactivator-1 α isoforms selectively regulate multiple splicing events on target genes. *J. Biol. Chem.* **291,** 15169–15184 (2016).

104. Monsalve, M. *et al.* Direct coupling of transcription and mRNA processing through the thermogenic coactivator PGC-1. *Mol. Cell* **6,** 307–316 (2000).

105. Ljubicic, V., Burt, M. & Jasmin, B. J. The therapeutic potential of skeletal muscle plasticity in Duchenne muscular dystrophy: Phenotypic modifiers as pharmacologic targets. *FASEB J.* **28,** 548–568 (2014).

106. Dial, A. G. *et al.* The role of AMP-activated protein kinase in the expression of the dystrophin-associated protein complex in skeletal muscle. *FASEB J.* fj.201700868RRR (2018). doi:10.1096/fj.201700868RRR

107. Charbonnier, F. Exercise-induced neuroprotection in SMA model mice: A means for determining new therapeutic strategies. *Molecular Neurobiology* **35,** 217–223 (2007).

108. Ng, S. Y., Manta, A. & Ljubicic, V. Exercise biology of neuromuscular disorders. *Appl. Physiol. Nutr. Metab.* 1–44 (2018).

109. McCartney, N., Moroz, D., Garner, S. H. & McComas, A. J. The effects of strength training in patients with selected neuromuscular disorders. *Med. Sci. Sport. Exerc.*

20, 362–368 (1988).

110. Lewelt, A. *et al.* Resistance strength training exercise in children with spinal muscular atrophy. *Muscle Nerve* **52,** 559–567 (2015).

111. Madsen, K. L. *et al.* Training improves oxidative capacity, but not function, in spinal muscular atrophy type III. *Muscle Nerve* **52,** 240–4 (2014).

112. Bora, G. *et al.* Effects of Arm Cycling Exercise in Spinal Muscular Atrophy Type II Patients: A Pilot Study. *J. Child Neurol.* 088307381775050 (2018). doi:10.1177/0883073817750500

113. Biondi, O. *et al.* Exercise-Induced Activation of NMDA Receptor Promotes Motor Unit Development and Survival in a Type 2 Spinal Muscular Atrophy Model Mouse. *J. Neurosci.* **28,** 953–962 (2008).

114. Chali, F. *et al.* Long-term exercise-specific neuroprotection in spinal muscular atrophy-like mice. *J. Physiol.* **594,** 1931–1952 (2016).

115. Grondard, C. *et al.* Regular Exercise Prolongs Survival in a Type 2 Spinal. *J. Neurosci.* **25,** 7615–7622 (2005).

116. Biondi, O. *et al.* Physical exercise reduces cardiac defects in type 2 spinal muscular atrophy-like mice. *J. Physiol.* **590,** 5907–25 (2012).

117. Biondi, O. *et al.* Exercise-induced activation of NMDA receptor promotes motor unit development and survival in a type 2 spinal muscular atrophy model mouse. *J. Neurosci.* **28,** 953–962 (2008).

118. Cerveró, C. *et al.* Chronic Treatment with the AMPK Agonist AICAR Prevents Skeletal Muscle Pathology but Fails to Improve Clinical Outcome in a Mouse Model of Severe Spinal Muscular Atrophy. *Neurotherapeutics* **13,** 198–216 (2016).

119. Farooq, F. *et al.* Celecoxib increases SMN and survival in a severe spinal muscular

atrophy mouse model via p38 pathway activation. *Hum. Mol. Genet.* **22,** 3415–3424 (2013).

120. Tadesse, H., Deschenes-Furry, J., Boisvenue, S. & Cote, J. KH-type splicing regulatory protein interacts with survival motor neuron protein and is misregulated in spinal muscular atrophy. *Hum. Mol. Genet.* **17,** 506–524 (2007).

Exercise-induced signaling in skeletal muscle of spinal muscular atrophy mice

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Abstract

Spinal muscular atrophy (SMA) is a health- and life-limiting neuromuscular disorder caused by the deficiency of survival motor neuron (SMN) protein. While historically considered a motoneuron disease, current understanding of SMA emphasizes the systemic nature of the condition, which requires addressing affected peripheral tissues, skeletal muscle in particular. Chronic physical activity is safe and modestly effective in SMA patients, however the underlying cellular events that drive beneficial physiological adaptations are undefined. Thus, the purpose of this study was to examine the effects of a single bout of physical activity on molecular mechanisms associated with adaptive remodelling in the skeletal muscle of *Smn*$^{2B/-}$ SMA-like mice. AMP-activated protein kinase (AMPK) and p38 mitogen-activated protein kinase (p38) expression and activity were not affected by SMA-like conditions, which suggests the maintenance of the molecular apparatus critical for exercise-induced plasticity. In response to a single bout of exercise, canonical responses such as AMPK, p38, and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) activation were preserved in *Smn*$^{2B/-}$ animals. Furthermore, molecules involved in SMN transcription including protein kinase B (AKT) and extracellular signal-regulated kinases (ERK)/ ETS-like gene 1 (ELK), were also altered following acute physical activity. Collectively, these changes were coincident with an exercise-evoked increase in full-length SMN expression. Acute exercise was also able to mitigate the aberrant autophagy program in the skeletal muscle of *Smn*$^{2B/-}$ mice. This study advances our understanding of the exercise biology of SMA and highlights the AMPK-p38-PGC-1α axis as a potential regulator of SMN expression along side AKT and ERK/ELK signalling.

Introduction

Spinal muscular atrophy (SMA) is a multi-system neuromuscular disorder (NMD) that is the leading genetic cause of infant mortality[1]. This life-limiting disease affects approximately one in every 10,000 live births and is the second most common autosomal recessive disorder following cystic fibrosis[2]. SMA is caused by a homozygous mutation in the survival motor neuron (SMN) 1 gene, which ablates its SMN protein product. As a result, individuals affected by SMA rely solely on the virtually identical SMN2 gene to produce SMN protein. However, 80-90% of the SMN transcripts from the SMN2 gene are truncated due to a single base substitution of exon 7. These truncated SMN transcripts, known as SMNΔ7, are translated into a rapidly degraded SMN protein. The remaining ~10-20% of SMN mRNA transcribed from SMN2 are full-length transcripts, which are synthesized to functional SMN protein.

The abundance of SMN expressed from SMN2 genes is the primary disease modifier in SMA[3]. Hence, many efforts in recent years emphasize increasing our understanding of the mechanisms that regulate SMN gene expression, and thus SMN protein content. For example, transcriptional factors such as ETS-like gene 1 (ELK) and cAMP response element-binding protein (CREB) have been shown to repress and drive SMN expression from SMN2, respectively[4,5]. In addition, cellular SMN levels are regulated by protein degradation processes, such as the ubiquitin-proteasome and autophagy systems, which serve to dismantle dysfunction molecules[6–8]. These transcriptional and post-translational mechanisms of SMN expression are dysregulated in SMA, the correction of which may serve as potential therapeutic targets to mitigate the SMA pathology[4,5,8,9]. For instance, promoting SMN gene expression, such as its increased transcription, enhanced mRNA stability or preventing its degradation, prolongs survival of

pre-clinical murine models of SMA[10–13]. While elevating SMN expression in central tissues, in particular the spinal cord and brain, improves health- and lifespan of SMA-like mice, the optimal mitigation of the pathology additionally requires augmented SMN levels in peripheral tissues, such as skeletal muscle[14,15]. Thus, identifying interventions that drive multisystem effects, as well as evoke gene expression at several phases (e.g., transcription, mRNA processing, post-translational modification), should be considered when designing therapeutic strategies for SMA.

Exercise is an affordable and accessible intervention that elicits favorable cellular and physiological adaptations in healthy individuals[16], as well as in those with chronic health disorders such as type 2 diabetes, cardiovascular disease, and cancer[16,17]. Chronic physical activity-induced adaptations in α-motoneurons (αMNs), neuromuscular junctions (NMJs), and skeletal myofibers enhance the robustness of the neuromuscular system[18–20]. For example, exercise training accelerates axonal regulatory and transport mechanisms[21,22], improves NMJ morphology[23,24], and enhances neurotransmission[25]. In the pre-clinical SMA-like context, these neuromuscular alterations, evoked either by transgenic[5], pharmacological[13,14,26], or physiological[4,5] means, attenuate disease phenotypes and dysfunction. Indeed, exercise training in SMA-like animals prolongs survival rate, diminishes muscle weakness and enhances motor behavior[27,28]. These training adaptations are driven, in part, by the stimulation SMN gene expression as well as through the induction of other, complementary neuroprotective mechansims[29]. This evidence is supported by recent studies that demonstrate physiological improvements in type 2 or 3 SMA patients who participated in chronic endurance- or resistance-type exercise protocols[28]. However, the underlying molecular mechanisms of exercise-induced adaptations in SMA remain undefined. Specifically, the intracellular signaling response

provoked by a single bout of exercise is completely unknown. These acute responses, which regulate gene expression, are in and of themselves insufficient to cause neuromuscular adaptations. It is only when these exercise bouts are repeated in a training regime lasting weeks of sufficient intensity that beneficial phenotypic plasticity is revealed[30]. Investigating these exercise-evoked cellular events in models of SMA will expand our knowledge of the biology of the disorder and may reveal novel pathways for further therapeutic pursuit.

The purpose of the present study was to investigate acute exercise-induced signaling in the skeletal muscle of *Smn*$^{2B/-}$ SMA-like animals, a relatively less severe model of SMA. To this end, we first surveyed skeletal muscle biology across a disease progression time course in order to determine the expression and activation of molecules important for maintaining and remodeling neuromuscular phenotype. Second, we examined the signaling cascades that are simulated by a single bout of endurance-type exercise, and whether these pathways are associated with the induction of SMN gene expression. We hypothesized that molecules important for governing muscle phenotype, such as adenosine monophosphate-activated protein kinase (AMPK), p38 mitogen-activated protein kinase (p38), unc-51 like autophagy activating kinase (ULK1), and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), will be altered during disease progression in SMA-like mice. We also postulated that a single bout of exercise would evoke favorable changes in myocellular signaling and gene expression, including those molecules and processes germane to SMN.

Methods

Animals. All mice were housed and cared for according to the Canadian Council on Animal Care guidelines in the McMaster Central Animal Facility. *Smn*$^{2B/-}$ mice (n = 12), which display a less severe SMA-like phenotype and extended lifespan relative to alternative murine models of SMA, such as the *SmnΔ7* and *Smn1*$^{C/C}$ mice[31,32], were utilized in these studies. Littermate *Smn*$^{2B/+}$ mice (n = 12) that do not express the SMA phenotype were used as healthy controls[33]. The mice were bred by crossing *Smn*$^{2B/2B}$ mice with heterozygous *Smn*$^{+/-}$ mice, similar to previous studies[34–36]. *Smn*$^{2B/2B}$ and *Smn*$^{+/-}$ mice were kind gifts from Dr. Rashmi Kothary, University of Ottawa and the Ottawa Hospital Research Institute. For the timecourse comparison between *Smn*$^{2B/+}$ and *Smn*$^{2B/-}$ mice, animals were euthanized by cervical dislocation and tissues were collected at a pre-symptomatic stage [postnatal day (P) 9], early symptomatic stage (P13), and late symptomatic stage (P21). The gastrocnemius (GAST), soleus (SOL), quadriceps (QUAD), tibialis anterior (TA), and extensor digitorum longus (EDL) muscles were harvested at these timepoints. GAST, QUAD, and TA muscles were immediately flash frozen in liquid nitrogen, while SOL muscles were mounted in optimum cutting temperature compound (Fisher Scientific, Hampton, US) and frozen in isopentane cooled in liquid nitrogen. All tissues were stored at -80 ^{o}C until analysis.

Acute exercise protocol. A cohort of *Smn*$^{2B/-}$ mice were randomly assigned to a sedentary group (*Smn*$^{2B/-}$ SED), 0 hrs post-exercise (*Smn*$^{2B/-}$ 0hr) or 3 hrs post-exercise (*Smn*$^{2B/-}$ 3hr) groups. A sedentary *Smn*$^{2B/+}$ group (*Smn*$^{2B/+}$ SED) was also included to serve as a healthy, non-exercised control. Animals in the *Smn*$^{2B/-}$ 0hr and 3hr groups were acclimatized at P15 and P16 to physical activity on a motorized treadmill (Columbus Instruments, Columbus, USA) for a duration of 5 min at a speed of 3m/min on each day.

On P17, the *Smn*$^{2B/-}$ animals were challenged to a constant 3m/min treadmill protocol at a 0^{o} incline until the inability to continue exercise was empirically determined. Specifically, the running endpoint was defined as when: 1) mice were no longer responsive to gentle mechanical prodding with a test tube cleaning brush, and subsequently; 2) the animal was not able to self-right within 30 sec when placed supine. Following the cessation of physical activity, animals in the *Smn*$^{2B/-}$ 0hr group were euthanized immediately, while the *Smn*$^{2B/-}$ 3hr animals were placed in a home cage for 180 min with access to water ad libitum. During this time, the *Smn*$^{2B/+}$ SED and *Smn*$^{2B/-}$ SED mice were killed and their tissues harvested. Mice in the *Smn*$^{2B/-}$ 3hr group were euthanized 180 min after exercise and muscles removed. Tissues were collected from all animals as described above. Healthy *Smn*$^{2B/+}$ mice were not challenged with the acute exercise stimulus, as the current study examines the impact of exercise in the context of SMA. There is an abundance of literature dedicated to exploring the effects of a single bout of physical activity on intracellular signaling and gene expression in the healthy condition in animal models [30,37] and human participants[38,39].

DNA isolation and genotyping. Mouse minimal ear or tail clippings were collected at P8. DNA was isolated by boiling the tissue in 50 mM NaOH for 30 min. 1M Tris HCl was added to neutralize the solution. For genotyping, the primers utilized were: F-TTGGCCTGAACTGCCAGCTGGCGCAGG, and R-TCCCGCAGCGCAG-ACCGTTTTCGCTCG (Sigma-Aldrich, Oakville, Canada). Polymerase chain reaction (PCR) was conducted with the following parameters: 94 ^{o}C for 3 min, 35 cycles at 94 ^{o}C for 1 min, 50 ^{o}C for 1 min, 72 ^{o}C for 1 min, followed by a 10 min extension at 72 ^{o}C.

Protein extraction and quantification. Frozen muscle was mechanically homogenized with a tissue pulverizer (Cellcrusher, Cork, Ireland) in a liquid nitrogen bath and placed in RIPA buffer (Sigma-Aldrich) supplemented with phosphatase and protease

inhibitors (Roche, Basel, Switzerland). The samples were sonicated 5 sec x 5 at maximum power. Samples were spun for 10 min at 14,000 x g and the supernatants were collected. A bicinchonic assay (Thermo Fisher Scientific, Burlington, ON, Canada) was conducted to determine protein concentrations.

Western blotting. QUAD muscle homogenate samples were loaded in 10-15% polyacrylamide gels and electrophoresed. Subsequently, proteins were transferred onto nitrocellulose membranes, which were stained with Ponceau S in order to verify equal loading across samples. Membranes were incubated with 5% bovine serum albumin (BSA) in Tris-buffered saline with 1% Tween-20 (TBST) for 1 hr at room temperature. Proteins were probed at 4 ^{o}C overnight with the following antibodies: AMPK (2535, Cell Signaling, Danvers, US), phosphorylated AMPK (pAMPK; 2535, Cell Signaling), p38 (9212, Cell Signaling), phosphorylated p38 (pp38; 9211, Cell Signaling), PGC-1α (AB3242, EMD Millipore, Burlington, US), SMN (610646, BD Biosciences, Mississauga, CA), CREB (MAB5432, EMD Millipore), phosphorylated CREB (pCREB; 06-519, EMD Millipore), ELK (sc-365876, Santa Cruz), phosphorylated ELK (pELK; sc-8406, Santa Cruz, Dallas, US), extracellular signal–regulated kinases 1 and 2 (ERK; 9101, Cell Signaling), phosphorylated ERK1/2 (pERK; 9102, Cell Signaling), protein kinase B (AKT; 4691, Cell Signaling), phosphorylated AKT (pAKT; 9271, Cell Signaling), ULK1 (D8H5, Cell Signaling), phosphorylated ULK1 (pULK1; 5869, Cell Signaling), sequestosome-1 (p62; P0067, Sigma Aldrich, St. Louis, US), and microtubule-associated protein 1A/1B-light chain 3 (LC3I/II; 4108, Cell Signaling). Membranes were washed in TBST 3 x 5 min and then incubated with the appropriate horseradish peroxidase-linked secondary antibodies for 70 min at room temperature. The membranes were visualized through enhanced chemiluminescence (Biorad) using a FluorChem SP Imaging System (Alpha Innotech

Corporation, San Leandro, USA). Densitometry was performed using Image Lab analysis (Biorad, Mississauga, Canada).

Immunofluorescence microscopy. SOL muscles stored in OCT were cut into 10 µm thick sections on a cryostat (Thermofisher Scientific) at -20 ºC. Prior to fixation, samples were air dried for ~30 min. Tissues were fixed with 4% paraformaldehyde for 10 min and then washed with PBS/Tween-20 (PBST). To avoid non-specific binding, the slides were incubated with 10% goat serum in 1% BSA for 90 min. Tissues were probed for PGC-1α (517380, Santa Cruz, Dallas, US) at 1:1,000 dilution in 1% BSA overnight at 4 ºC. The following day, slides were washed with PBST and incubated in Alexa-conjugated secondary (Thermo Fisher Scientific) at 1:500 in 1% BSA for 2 hrs at room temperature, followed by another 3 x 5 min wash in PBST. Slides were then fixed again in 4% paraformaldehyde followed by a PBST wash. Slides were then incubated in 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI; Thermo Fisher Scientific) at 1:20,000 in 1% BSA for 10 min to label myonuclei. After slides were dried, fluorescent mounting media (Dako Agilent Technologies, Mississauga, Canada) was applied and the slide was mounted with a coverslip. Imaging was done on the Nikon Eclipse 90i microscope at a magnification of 20x and captured with a photometric Cool SNAP HQ2 Fluorescent camera (Nikon instruments, Netherlands). The subcellular localization method has been previously described[40]. After determining the total cross sectional area of the muscle, multiple regions of interest were generated to represent 35-40% of the muscle. In order to avoid subjectivity from the evaluator, subject groups were blinded to the rater prior to analyses. A binary layer was set to represent myonuclei with the DAPI stain. Myonuclear PGC-1α localization was then determined as the percentage of PGC-1α fluorescence,

measured by sum intensity, overlaid with the DAPI binary layer. The remaining PGC-1α fluorescence was considered cytosolic.

RNA isolation, reverse transcription, quantitative real-time PCR (qPCR), and endpoint PCR. Total RNA was isolated from TA muscles. 1 ml of TRIzol reagent (Invitrogen, Carlsbad, US) was used to homogenize all muscle samples in Lysing D matrix tubes (MP Biomedicals, Santa Ana, US) with the FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals) for 40 sec at a speed of 6.0 m/s. Homogenized muscles were mixed in 200 µl of chloroform (Thermo Fisher Scientific) and shaken vigorously for 15 sec then centrifuged at 12,000 g for 10 min. The upper aqueous layer (RNA) was purified by the Total RNA Omega Bio-Tek kit (VWR International, Radnor, US). Concentration and purity of the RNA was determined using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). RNA samples were then reverse-transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) according to the instructions provided by the manufacturer. All qPCR assays were run with 2 µg of cDNA in triplicate 6 µl reactions containing GoTaq qPCR Master Mix (Promega, Madison, US). Data was analyzed using the comparative C_T method[41]. Ribosomal protein S11 (RPS11) was used as the normalizing gene since its C_T values not differ between *Smn*$^{2B/-}$ and *Smn*$^{2B/+}$ groups, as well as during the developmental timecourse and after acute exercise (data not shown). qPCR primers (Sigma Aldrich, St. Louis, US) utilized were as follows: PGC-1α F-AGTGGTGTAGCGACCAAT, R-GGGCAATCCGTCTTCATCCA; SMN F-TCCTTCAGGACCACCAATA, R-CCACTGATGACGAGGAGACG; ULK1 F-GCTCCGGTGACTTACAAAGCTG, R-GCTGACTCCAAGCCAAAGCA; p62 F-CCCAGTGTCTTGGCATTCTT, R-AGGGAAAGCAGAGGAAGCTC; BCL2/adenovirus E1B 19 kDa protein-interacting

protein 3 (Bnip3) F-TTCCACTAGCACCTTCTGATGA, R-GAACACCGCATTTACAGAACAA; beclin-1-associated autophagy-related key regulator (ATG14) F- AGCGGTGATTTCGTCTATTTCG, R-GCTGTTCAATCCTCATCTTGCAT; GABA receptor-associated protein-like 1 (Gabarap1) F- CATCGTGGAGAAGGCTCCTA, R-ATACAGCTGGCCCATGGTAG; RPS11 F- CGTGACGAACATGAAGATGC, R- GCACATTGAATCGCACAGTC.For endpoint PCR detection of SMN and SMNΔ7 mRNAs, 1 µg of cDNA was added to a reaction mix containing Taq polymerase and primers. Here, the SMN primers (Sigma Aldrich) were F-CTGATGCCCTGGGCAGTATGCTA, R-CCACTGATGACGAGGAGACG. PCR products were resolved on a 4% agarose gel at 110 mV for 60 min. The percentage of misspliced mRNA transcripts was determined using Image Lab analysis (Biorad), where the intensity of the truncated SMNΔ7 band was determined relative to the total band intensities of both the full-length SMN and SMNΔ7 bands.

Statistics. Two-way analysis of variance (ANOVA) and one-way ANOVA were employed to compare means between experimental groups, as appropriate. Specifically, the two-way ANOVA was employed to examine data in the disease progression experiments, whereas the one-way ANOVA was utilized for the acute exercise experiments. Statistical analyses were performed with the Graphpad Prism software package (GraphPad Software, La Jolla, US). Data are presented as mean ± SEMs.

Results

Gross characterization of type 2 SMA-like mice during disease progression. We first examined whole body mass and muscle weights of the *Smn*$^{2B/-}$ mice and their healthy

Smn$^{2B/+}$ littermates at presymptomatic (P9), early symptomatic (P13), and late symptomatic (P21) stages. *Smn*$^{2B/-}$ animal weights (P9, 4.4 ± 0.1 g; P13, 7.2 ± 0.4 g) were comparable to the healthy *Smn*$^{2B/+}$ mice (P9, 5.7 ± 0.7 g; P13, 8.1 ± 0.6 g) at P9 and P13 (Figure 1A). At P21, *Smn*$^{2B/+}$ mice had a mass of 9.3 g ± 0.4 g while the *Smn*$^{2B/-}$ animals weighed ~50% less (4.4 ± 0.2 g; $P < 0.05$). Qualitative assessment also revealed gross morphological differences between the two genotypes at P21 (Figure 1B). Similarly, the TA, GAST, and QUAD muscle masses were significantly lower (~60-70%) at the late symptomatic stage in *Smn*$^{2B/-}$ animals compared to the *Smn*$^{2B/+}$ group (Figure 1C). EDL and SOL muscle weights were similar between genotypes at all timepoints, possibly due, in part, to detection limitations at such small values. A markedly lower SMN protein level in the QUAD muscles of *Smn*$^{2B/-}$ mice at P21 was confirmed via Western blot analysis (Figure 1D).

AMPK, p38, and PGC-1α levels in the skeletal muscle of SMA-like mice. We next examined the AMPK-p38-PGC-1α signaling axis in the QUAD muscles of *Smn*$^{2B/+}$ and *Smn*$^{2B/-}$ animals throughout the experimental timecourse. The activation status of AMPK (i.e., the phosphorylated form of the protein relative to the total amount of the enzyme) tended to be higher in *Smn*$^{2B/-}$ animals compared to *Smn*$^{2B/+}$ mice at the P21 timepoint ($P = 0.11$; Figure 2A, 2B). The activation status of p38 was similar between genotypes at all timepoints (Figure 2A, 2C). Myocellular PGC-1α protein content in *Smn*$^{2B/-}$ mice was comparable to their *Smn*$^{2B/+}$ littermates at presymptomatic and symptomatic stages (Figure 2A, 2D). In contrast, at P21 PGC-1α expression was significantly lower (-35%) in *Smn*$^{2B/-}$ mice versus the *Smn*$^{2B/+}$ group.

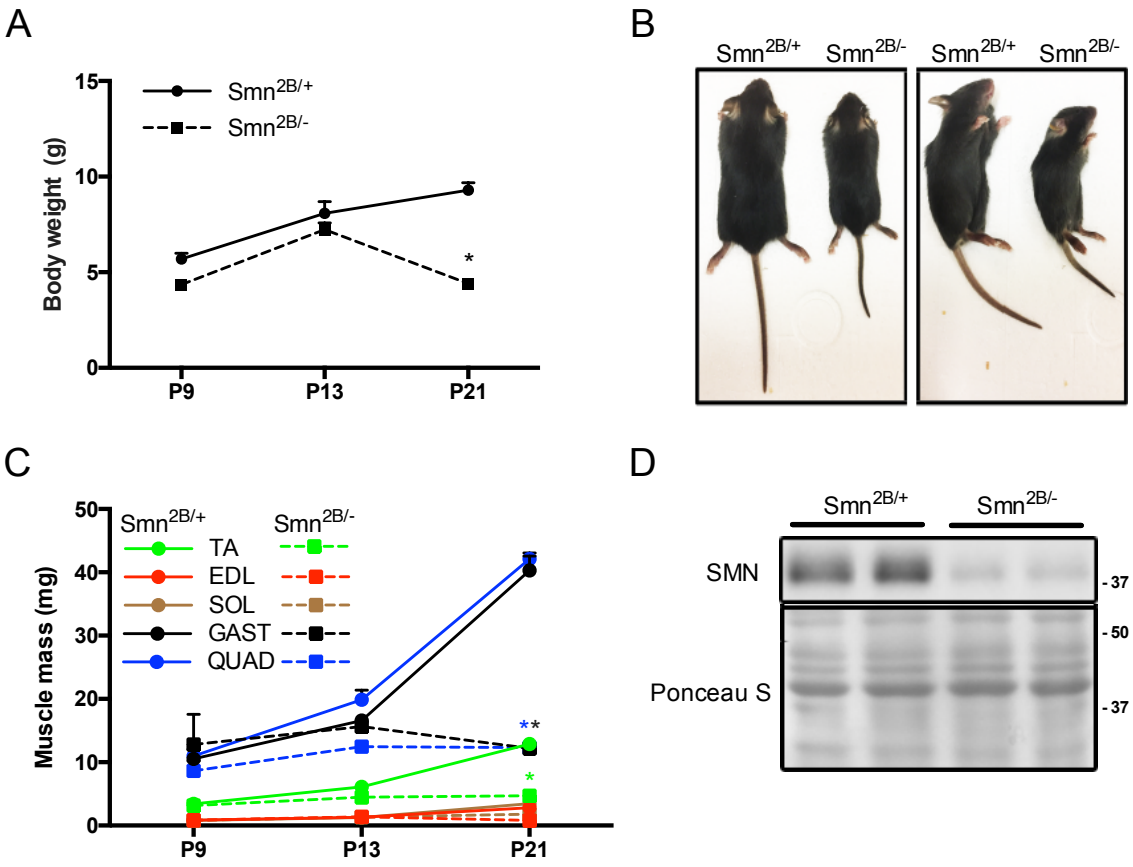

Figure 1. Characterization of *Smn$^{2B/-}$* mice during disease progression. *A*, Body mass of *Smn$^{2B/+}$* and *Smn$^{2B/-}$* mice at postnatal day (P) 9, P13, and P21. *B, Smn$^{2B/+}$* and *Smn$^{2B/-}$* mice in prone (left panel) and side-lying (right panel) positions. *C*, Mass of the tibialis anterior (TA; Green), extensor digitorum longus (EDL; Red), soleus (SOL, Brown), gastrocnemius (GAST, Black), and quadriceps (QUAD, Blue) muscles from *Smn$^{2B/-}$* mice and *Smn$^{2B/+}$* littermates. *D,* Representative Western blot of survival motor neuron (SMN) protein in QUAD muscles of *Smn$^{2B/+}$* and *Smn$^{2B/-}$* mice. Ponceau S stain is displayed below to indicate equal loading between samples. Ladder markers are expressed as kDa. *, $P < 0.05$ vs. age-matched *Smn$^{2B/+}$*; n = 10.

Exercise-induced AMPK-p38-PGC-1α signaling. Alterations in the AMPK-p38-PGC-1 signaling cascade in *Smn*$^{2B/-}$ skeletal muscle prompted us to question whether these exercise-inducible pathways could be activated in response to physical activity. Hence, *Smn*$^{2B/+}$ and *Smn*$^{2B/-}$ mice at P17 were challenged with a single bout of exercise and intracellular signaling and gene expression analyses were subsequently performed. We selected P17 as the experimental timepoint here for two reasons: first, the *Smn*$^{2B/-}$ animals are firmly within the window of the disease phenotype, and second; the mice are mature enough to perform the exercise. As expected, although matched for relative workload (i.e., *Smn*$^{2B/+}$ and *Smn*$^{2B/-}$ mice ran until the inability to continue exercise was determined), in absolute terms the *Smn*$^{2B/-}$ mice ran significantly less than their *Smn*$^{2B/+}$ counterparts (*Smn*$^{2B/+}$, 424.4 m ± 43.46 m; *Smn*$^{2B/-}$, 64.05 m ± 9.59 m; data not shown). Sedentary, non-exercised *Smn*$^{2B/+}$ mice (*Smn*$^{2B/+}$ SED) acted as healthy, resting controls, while the *Smn*$^{2B/-}$ SED group served as non-exercised, SMA-like controls. Consideration was given to the sensitive, temporal nature of exercise-induced signaling by including a group of exercised *Smn*$^{2B/-}$ mice that were killed and whose QUAD, TA, and SOL muscles were harvested immediately post-exercise (*Smn*$^{2B/-}$ 0hr) to provide an impression of signal transduction during physical activity, as well as a *Smn*$^{2B/-}$ 3hr group that was killed 3 hours post-exercise, which represents a recovery state within the contracting tissues.

In QUAD muscles, phosphorylated AMPK levels in the *Smn*$^{2B/-}$ 0hr group were 1.9-2-fold greater ($P < 0.05$) than in the *Smn*$^{2B/+}$ SED and *Smn*$^{2B/-}$ SED groups (Figure 3A, 3B). AMPK activation status was also significantly higher (~2.4-fold) in *Smn*$^{2B/-}$ 0hr mice relative to the *Smn*$^{2B/+}$ SED and *Smn*$^{2B/-}$ SED groups, while AMPK activation in the *Smn*$^{2B/-}$ 3hr animals was 1.9-fold greater ($P < 0.05$) compared to the *Smn*$^{2B/+}$ SED and

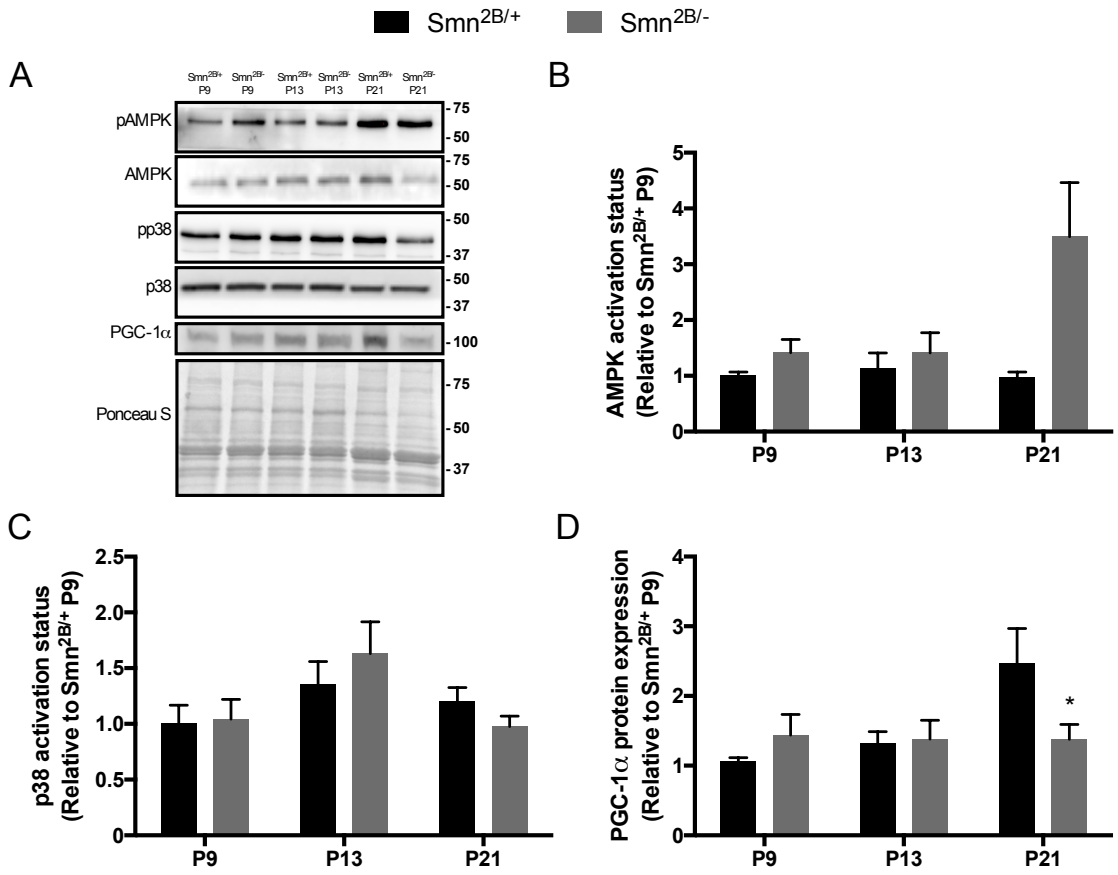

Figure 2. AMP-activated protein kinase (AMPK), p38 mitogen-activated protein kinase (p38), and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) levels in skeletal muscle of SMA-like mice. *A*, Representative Western blots of the phosphorylated form of AMPK (pAMPK), total AMPK, phosphorylated form of p38 (pp38), total p38, and PGC-1α in QUAD muscles of *Smn*$^{2B/+}$ and *Smn*$^{2B/-}$ animals at presymptomatic (P9), early symptomatic (P13), and late symptomatic timepoints (P21). Ponceau S stain is shown below. Approximate molecular weight markers (kDa) are denoted at right of blots. Graphical summaries of AMPK activation status (i.e., the phosphorylated form of the protein relative to its total amount; *B*), p38 activation status (*C*), and PGC-1α protein expression (*D*) in the QUAD muscles of *Smn*$^{2B/+}$ and *Smn*$^{2B/-}$ animals at P9, P13, and P21. Values are displayed as a fold difference relative to P9 *Smn*$^{2B/+}$ animals. *, $P < 0.05$ vs. age-matched *Smn*$^{2B/+}$; n = 8.

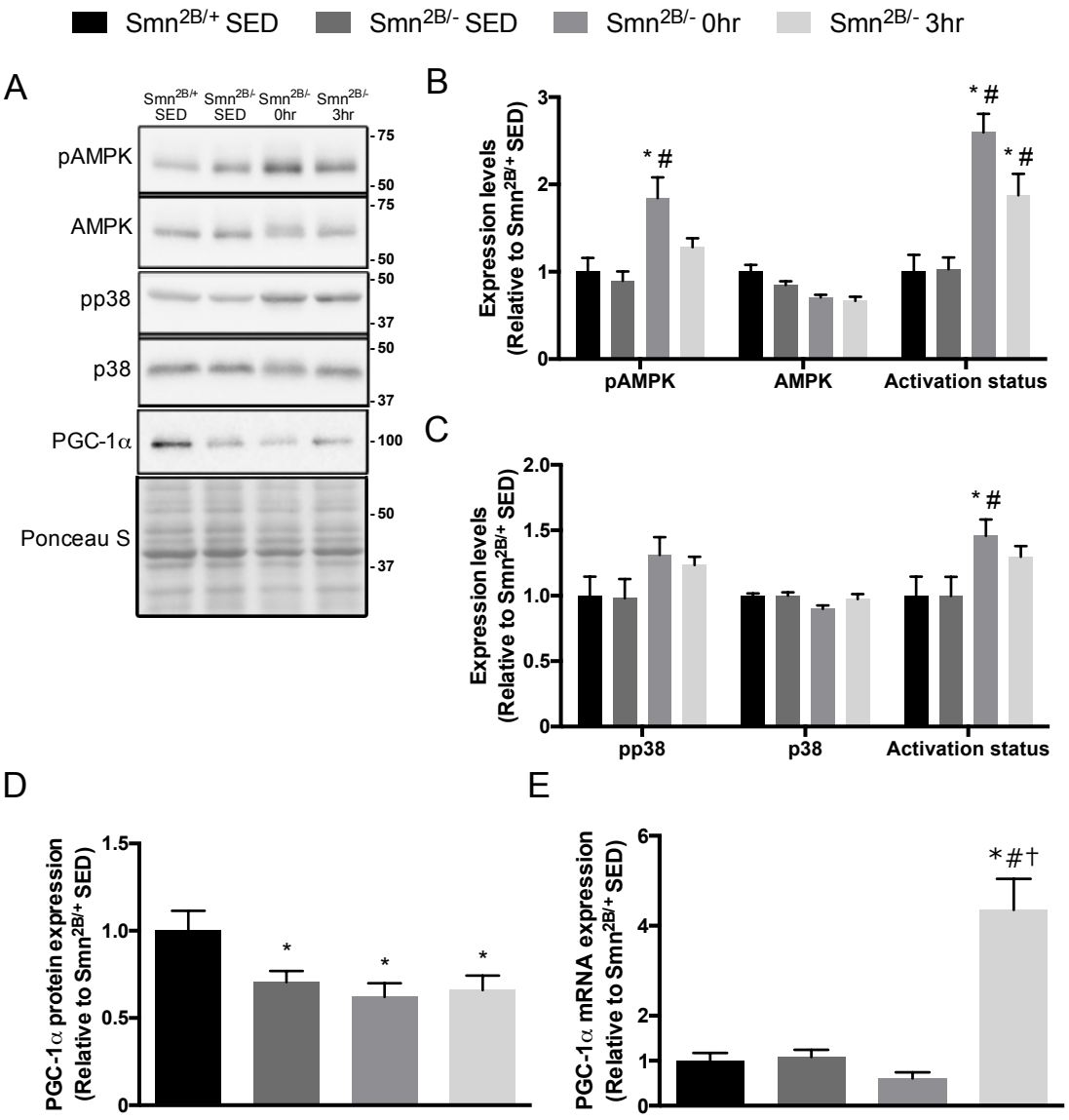

Figure 3: Exercise-induced signaling in type 2 SMA-like skeletal muscle. *A*, Representative Western blots of pAMPK, AMPK, pp38, p38 and PGC-1α in QUAD muscles of sedentary *Smn*$^{2B/+}$ mice (*Smn*$^{2B/+}$ SED), sedentary *Smn*$^{2B/-}$ mice (*Smn*$^{2B/-}$ SED), and *Smn*$^{2B/-}$ animals that were killed 0 hours post-exercise (*Smn*$^{2B/-}$ 0hr), and 3 hours post-exercise (*Smn*$^{2B/-}$ 3hr). A Ponceau S stain is also displayed below to demonstrate equal loading across samples. Ladder markers are expressed as kDa. Graphical summaries of pAMPK, AMPK, and AMPK activation status (*B*), pp38, p38, and p38 activation status (*C*), and total myocellular PGC-1α levels (*D*) are shown. PGC-1α mRNA expression (*E*) in the TA muscles from all experimental groups. Values are expressed relative to *Smn*$^{2B/+}$ SED. *, $P < 0.05$ vs. *Smn*$^{2B/+}$ SED; #, $P < 0.05$ vs. *Smn*$^{2B/-}$ SED; †, $P < 0.05$ vs. *Smn*$^{2B/-}$ 0hr; $n = 7$.

Smn$^{2B/-}$ SED groups. Similarly, p38 activation status was ~50% greater ($P < 0.05$) in the *Smn*$^{2B/-}$ 0hr group relative to SED mice (Figure 3A, 3C). PGC-1α protein expression was significantly lower in *Smn*$^{2B/-}$ mice versus their *Smn*$^{2B/+}$ counterparts, and was unaltered with exercise or recovery (Figure 3A, 3D). PGC-1α mRNA expression in the TA muscle was similar between genotypes at rest and was elevated ~5-fold ($P < 0.05$) in *Smn*$^{2B/-}$ 3hr animals, as compared to all other experimental groups (Figure 3E).

Subcellular localization of PGC-1α in the skeletal muscle SMA-like animals with exercise. To investigate potential mechanisms responsible for the exercise-induced elevation in PGC-1α mRNA in the skeletal muscle of *Smn*$^{2B/-}$ animals, we next examined the subcellular localization of the enzyme. Indeed, it is well established that the myonuclear presence of this protein induces muscle remodeling via transcriptional and post-transcriptional events, in part via autoregulation[40,42]. We therefore performed immunofluorescence assays to detect myonuclear versus cytosolic PGC-1α abundance in the SOL muscles of mice from the four experimental groups. The majority of the protein was found within the cytosolic compartment relative to myonuclei (Figures 4A, 4B). Myonuclear PGC-1α localization was 35-40% greater ($P < 0.05$) 3-hours post-exercise in *Smn*$^{2B/-}$ mice relative to both SED groups (Figure 4A, 4B).

Expression and activation of SMN transcriptional regulators. Recent work from Frédéric Charbonnier's laboratory and others have identified the AKT-CREB and ERK-ELK signaling pathways as potent positive and negative regulators of SMN transcription, respectively[5, 45]. Thus, we examined the impact of acute exercise on the potential transcriptional control of SMN gene expression by analyzing the content and activity of these molecules. AKT phosphorylation and activation in QUAD muscles were similar between *Smn*$^{2B/+}$ SED

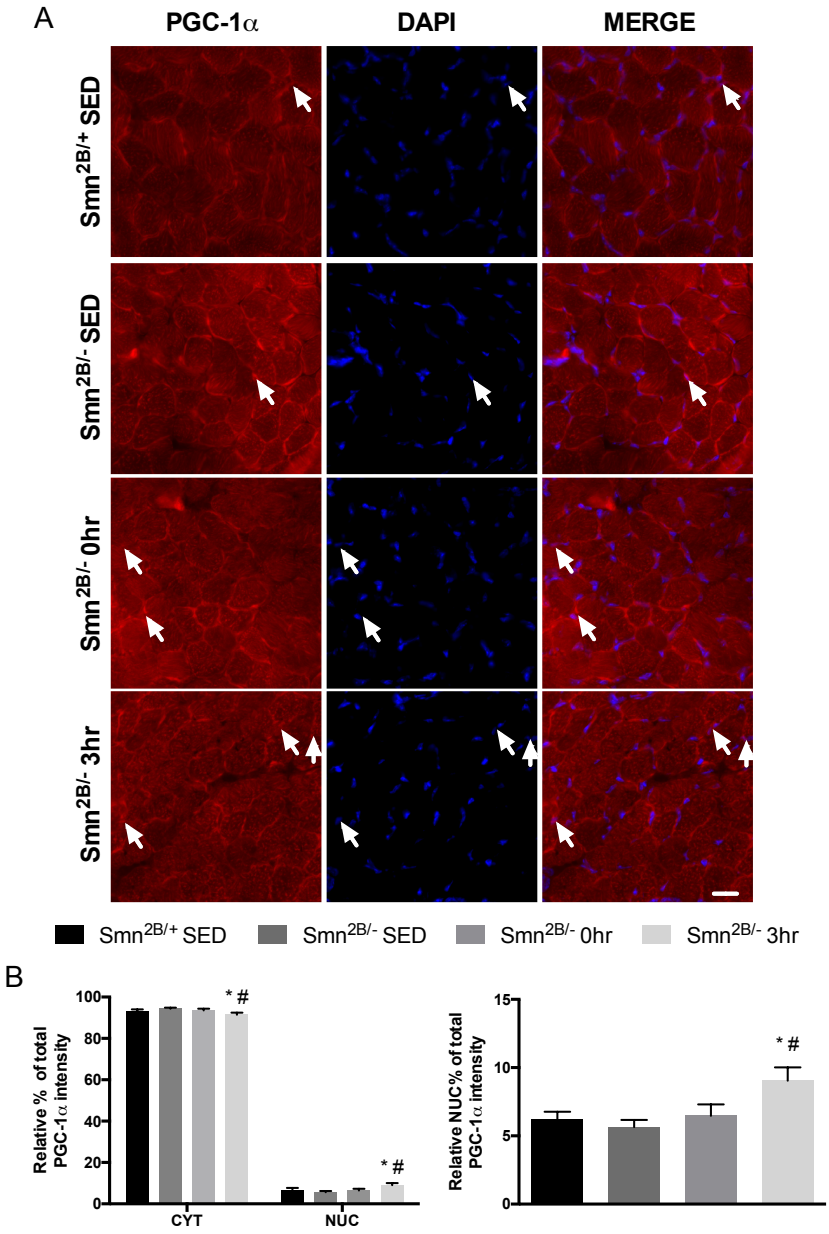

Figure 4: Subcellular localization of PGC-1α in the muscle of exercised SMA-like mice. *A,* Immunofluorescence images of PGC-1α in SOL muscles of *Smn*$^{2B/+}$ SED, *Smn*$^{2B/-}$ SED, *Smn*$^{2B/-}$ 0hr, and *Smn*$^{2B/-}$ 3hr mice. 6-diamidino-2-phenylindole dihydrochloride (DAPI) denotes myonuclei. *B left*, Graphical summary of PGC-1α subcellular localization in cytosolic (CYT) and nuclear (NUC) compartments of SOL muscles from the four experimental groups. *B, right*, Enlarged summary of the % NUC accumulation of PGC-1α from *B, left*. White arrows indicate PGC-1α present in myonuclei. *, $P < 0.05$ vs. *Smn*$^{2B/+}$ SED; #, $P < 0.05$ vs. *Smn*$^{2B/-}$ SED; $n = 7$.

and *Smn*$^{2B/-}$ SED groups, whereas the phosphorylation levels and activation status were significantly higher in *Smn*$^{2B/-}$ 0hr mice relative to *Smn*$^{2B/-}$ SED animals (Figure 5A, 5B). Total AKT levels were similar between the four experimental groups. CREB protein content was significantly lower in all *Smn*$^{2B/-}$ groups versus *Smn*$^{2B/+}$ SED mice (Figure 5A, 5C). This in turn contributed to the elevated CREB activation status in *Smn*$^{2B/-}$ animals relative to their *Smn*$^{2B/+}$ SED littermates. CREB signaling was unaffected by exercise or recovery in *Smn*$^{2B/-}$ mice. ERK phosphorylation and activation status were elevated by 60-70% ($P < 0.05$) in the *Smn*$^{2B/-}$ SED mice relative to *Smn*$^{2B/+}$ SED animals (Figure 5A, 5D). ERK phosphorylation and activation were completely normalized by exercise. Total ERK protein content was similar between groups. ELK phosphorylation levels were significantly higher (+2-fold) in the *Smn*$^{2B/-}$ SED and *Smn*$^{2B/-}$ 0hr groups compared to their *Smn*$^{2B/+}$ SED littermates (Figure 5A, 5E). ELK phosphorylation was completely normalized in *Smn*$^{2B/-}$ 3hr animals. Total ELK protein content was similar across all experimental groups. Finally, ELK activation status in *Smn*$^{2B/-}$ 3hr mice was significantly lower compared to the *Smn*$^{2B/+}$ SED, *Smn*$^{2B/-}$ SED, and *Smn*$^{2B/-}$ 0hr groups.

Exercise-induced SMN expression in the skeletal muscle of SMA-like animals. Some studies demonstrate that chronic physical activity elicits significant induction of SMN expression in SMA-like animals coincident with functional physiological improvements[5,27], while others do not[44]. These discordant results between studies may be due to differences in the SMA-like animal models utilized, exercise protocols, and tissues analyzed. To further investigate the possible mechanistic basis for exercise-mediated SMN induction, we asked whether a single bout of activity is capable of altering SMN gene expression in the skeletal muscle of *Smn*$^{2B/-}$ mice. Western blotting, as well as endpoint PCR and RT-qPCR analyses were utilized in order to address this question.

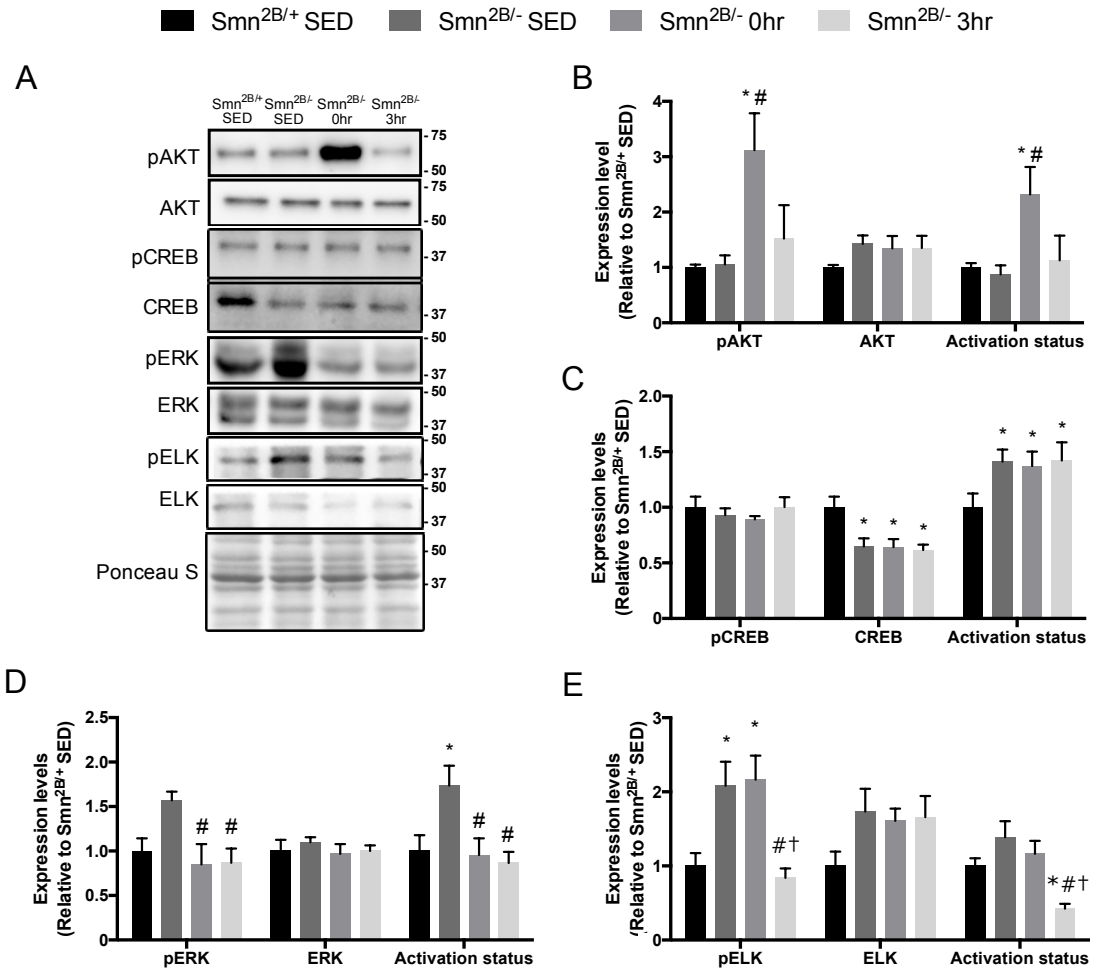

Figure 5: Exercise-induced expression and activity of SMN transcriptional regulators in the skeletal muscle of SMA-like mice. *A*, Representative Western blots of the phosphorylated form of protein kinase B (pAKT), total AKT, the phosphorylated form of cAMP response element-binding protein (pCREB), CREB, the phosphorylated form of extracellular signal-regulated kinase (pERK), ERK, as well as the phosphorylated form of ETS-like gene 1 (pELK) and ELK in QUAD muscles of *Smn*$^{2B/+}$ SED, *Smn*$^{2B/-}$ SED, *Smn*$^{2B/-}$ 0hr, and *Smn*$^{2B/-}$ 3hr animals. A Ponceau S stain is shown below that demonstrates equal loading between samples. Protein ladder markers at right of blots are expressed as kDa. Protein expression and activation status of AKT (*B*), CREB (*C*), ERK (*D*), and ELK (*E*) are graphically summarized. Values are expressed relative to *Smn*$^{2B/+}$ SED. *, $P < 0.05$ vs. *Smn*$^{2B/+}$ SED; #, $P < 0.05$ vs. *Smn*$^{2B/-}$ SED; †, $P < 0.05$ vs. *Smn*$^{2B/-}$ 0hr; n = 7.

SMN protein content in QUAD muscles, which was significantly lower in the *Smn*$^{2B/-}$ animals compared to *Smn*$^{2B/+}$ SED mice as expected, did not change with an acute bout of exercise or during post-exercise recovery (Figure 6A). Similarly, the abundance of full-length SMN transcripts was significantly lower in the TA muscles of *Smn*$^{2B/-}$ mice versus their *Smn*$^{2B/+}$ SED littermates, as revealed by endpoint PCR analyses (Figure 6B). However, the % inclusion of SMN exon 7 was increased by 40% ($P < 0.05$) in muscles from *Smn*$^{2B/-}$ 0hr mice versus their *Smn*$^{2B/-}$ SED counterparts. Consistent with the preceding protein and mRNA data, RT-qPCR results demonstrated significantly lower levels of full-length SMN transcripts in the TA muscles of all *Smn*$^{2B/-}$ groups, as compared to the *Smn*$^{2B/+}$ SED animals (Figure 6C). Full-length SMN transcript content was ~2.fold higher ($P < 0.05$) in the *Smn*$^{2B/-}$ 3hr mice relative to the *Smn*$^{2B/-}$ SED group ($P < 0.05$).

Acute exercise-induced autophagic signaling in SMA-like mice. Autophagy is an essential regulatory process involved in organelle disposal and recycling, which is dysfunctional in SMA animals[8,9]. Normalizing autophagy in SMA mitigates disease pathology[9]. Since a single bout of exercise initiates the autophagy program in healthy the healthy condition[43], we sought to investigate the effects of acute physical activity on autophagy gene expression in SMA-like skeletal muscle. Phosphorylated ULK1 protein levels and activation status in QUAD muscles were 2-2.2-fold greater ($P < 0.05$) immediately after exercise in *Smn*$^{2B/-}$ mice relative to *Smn*$^{2B/+}$ SED mice (Figure 7A, 7B). The expression of p62, a protein related to autophagosome formation, tended to be higher ($P = 0.07$) in the *Smn*$^{2B/-}$ SED group compared to *Smn*$^{2B/+}$ SED animals, while p62 levels were similar ($P > 0.05$) between the exercised *Smn*$^{2B/-}$ mice and *Smn*$^{2B/+}$ SED mice (Figure 7A, 7C). The LC3II:LC3I ratio was significantly greater

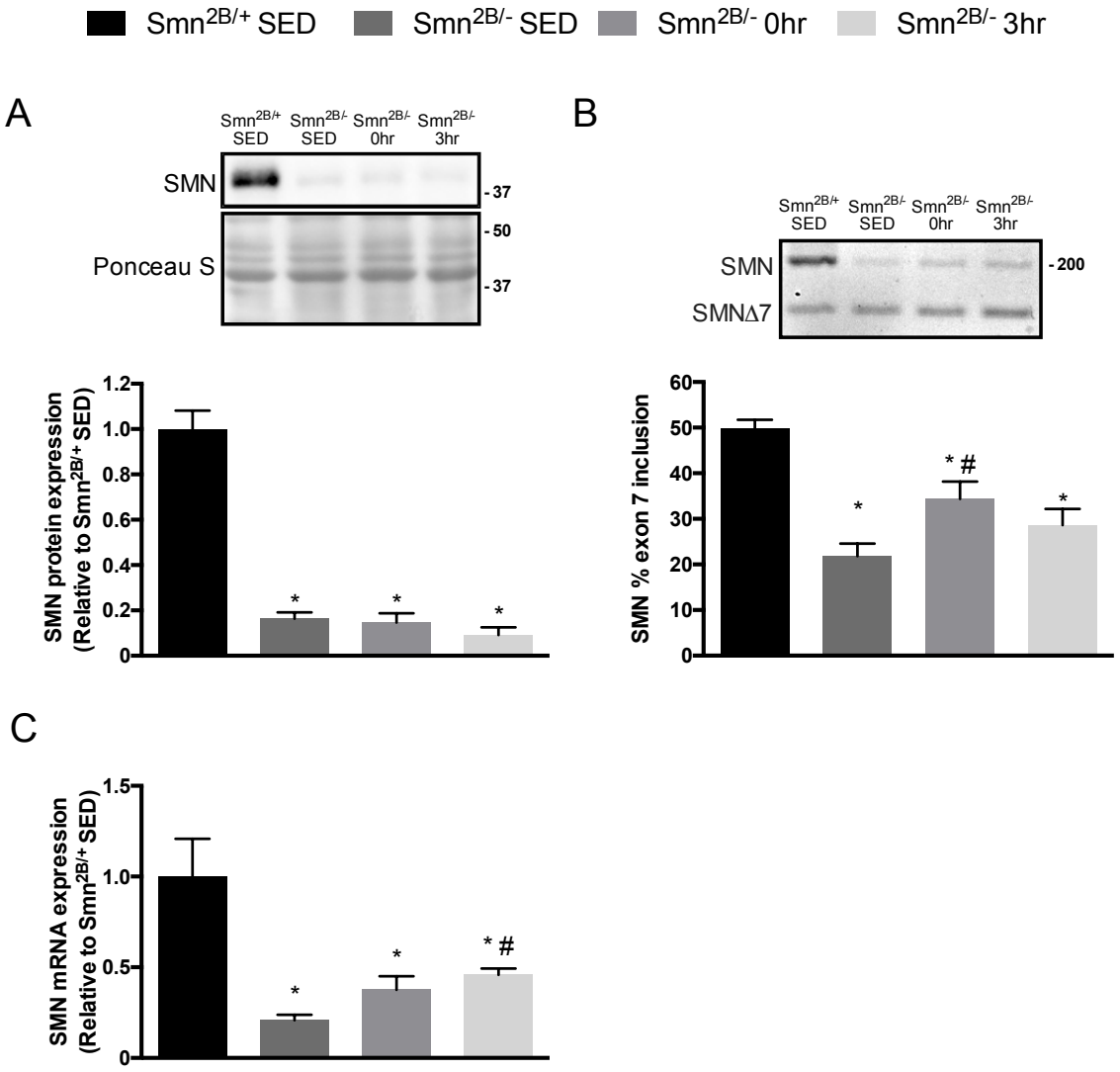

Figure 6: Exercise-induced SMN gene expression in skeletal muscle. *A,* Western blot of SMN protein content in QUAD muscles of *Smn*$^{2B/+}$ SED, *Smn*$^{2B/-}$ SED, *Smn*$^{2B/-}$ 0hr and *Smn*$^{2B/-}$ 3hr mice. Ponceau S stain and graphical summary are shown below. Ladder markers are expressed as kDa. Values are expressed relative to *Smn*$^{2B/+}$ SED. *B,* Representative endpoint PCR gel of the full length SMN mRNA (SMN) and the alternatively spliced SMN mRNA lacking exon 7 (SMNΔ7) in TA muscles. DNA ladder marker is at right of gel. Graphical summary is shown below. *C,* Summary of TA muscle SMN mRNA expression, as determined using real-time quantitative PCR analysis, in the four experimental cohorts. *, $P < 0.05$ vs. *Smn*$^{2B/+}$ SED; #, $P < 0.05$ vs. *Smn*$^{2B/-}$ SED; n = 9.

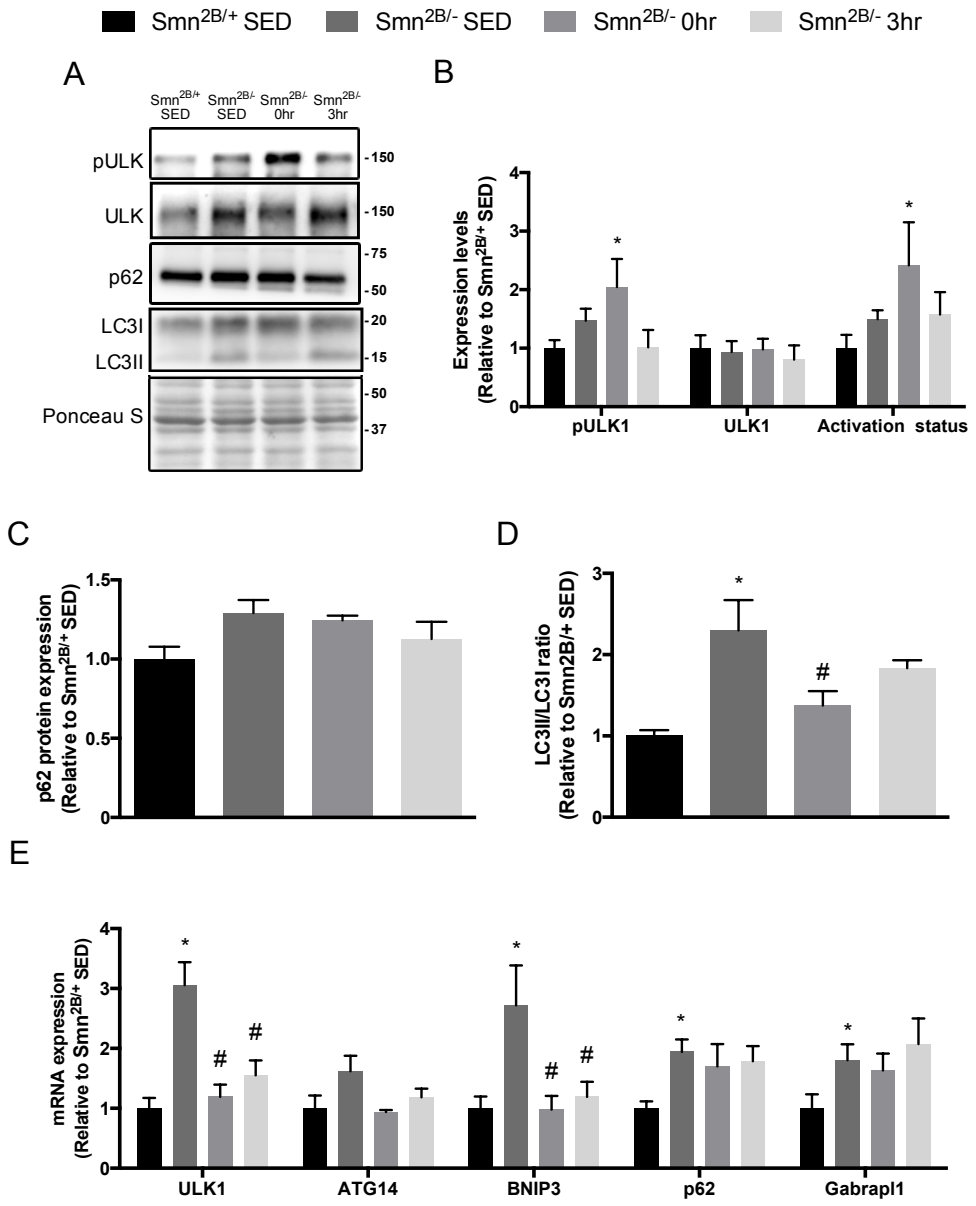

Figure 7: Exercise-induced autophagic signalling in the skeletal muscle of SMA-like animals. *A*, Representative Western blots of the phosphorylated form of unc-51-like autophagy-activating kinase 1 (pULK1), total ULK1, p62/Sequestosome-1 (p62), as well as the cytosolic LC3 (i.e., LC3I) and membrane bound LC3 (i.e., LC3II) in QUAD muscles of *Smn*$^{2B/+}$ SED, *Smn*$^{2B/-}$ SED, *Smn*$^{2B/-}$ 0hr, and *Smn*$^{2B/-}$ 3hr animals. A Ponceau S stain, indicative of equal loading between samples, is also displayed below. Approximate molecular weight markers (kDa) are denoted at left of blots. Protein expression and activation status of ULK1 (*B*), as well as the levels of p62 (*C)*, and the LC3II:LC3I ratio (*D*) are graphically summarized. *E*, Summaries of ULK1, autophagy-related gene 14 (ATG14), BCl2/adenovirus EIB 19 kda (BNIP3), p62, and GABA receptor-associated protein-like 1 (Gabrapl1) mRNA expression in TA muscles from mice in the four experimental groups. All data are expressed relative to *Smn*$^{2B/+}$ SED. *, $P < 0.05$ vs. *Smn*$^{2B/+}$ SED; #, $P < 0.05$ vs. *Smn*$^{2B/-}$ SED; n = 7.

(+2.3-fold) in the *Smn*$^{2B/-}$ SED mice versus their *Smn*$^{2B/+}$ SED littermates (Figure 7A, 7D). In *Smn*$^{2B/-}$ 0hr mice, this ratio of lipidated to unlipidated LC3 protein was significantly lower relative to *Smn*$^{2B/-}$ SED animals, and not different compared to the *Smn*$^{2B/+}$ SED group. To complement these protein analyses, we also assessed the effect of exercise and recovery on the abundance of mRNAs representative of the autophagy program. In TA muscles, ULK1, BNIP3, p62, and Gabrapl1 transcript levels in the *Smn*$^{2B/-}$ SED group were significantly elevated 2-3-fold relative to their *Smn*$^{2B/+}$ SED counterparts (Figure 7E). Furthermore, ULK1 and BNIP3 mRNA abundance was significantly reduced by exercise in both *Smn*$^{2B/-}$ groups and were comparable to *Smn*$^{2B/+}$ SED levels. In contrast, ATG14 mRNA expression was similar between genotypes and was not impacted by exercise or recovery.

Discussion

The purpose of the present study was to determine exercise-induced signaling cascades in the skeletal muscle of *Smn*$^{2B/-}$ SMA-like animals. Our data demonstrate that the expression and activity of molecules involved in maintaining and remodelling neuromuscular phenotype, including AMPK and p38 were unchanged in skeletal muscle during the manifestation and progression of SMA-like symptoms, while others such as PGC-1α were depressed coincident with increased disease severity. Similar to previous studies in healthy animals and humans[46], these proteins along with the autophagy regulator ULK1 were activated following an acute bout of endurance-type exercise in *Smn*$^{2B/-}$ mice. This suggests that canonical exercise-sensitive pathways involving AMPK, ULK1, p38, and PGC-1α are important for stimulating therapeutic plasticity of the neuromuscular system in SMA. Moreover, the chronic activation of these molecules may contribute to the

salutary exercise training-induced adaptations observed in alternative pre-clinical SMA models, as well as in SMA patients[28]. We also demonstrated that a single bout of exercise affects AKT-CREB and ERK-ELK signaling cascades that are associated with enhanced transcription of the SMN gene, which occurred coincident with elevations in full-length SMN mRNA expression. While certainly these acute responses to a single bout of exercise are in of themselves insufficient to alter SMA progression or severity, these data identify novel cellular and molecular mechanisms that drive corrective remodeling of the disease phenotype and thus highlight future therapeutic avenues for SMA.

AMPK, p38, and PGC-1α mediate many of the acute cellular responses and chronic adaptations to exercise[17]. Furthermore, the evidence indicates that these molecules also play important roles in SMA biology[10,26,28,40,47]. However, our understanding of the expression and function of these proteins in the SMA context is still being established. We therefore characterized AMPK, p38, and PGC-1α levels in the skeletal muscle of SMA-like mice across a timecourse of disease progression. Unexpectedly, AMPK and p38 content and activation were similar between healthy *Smn*$^{2B/+}$ mice and their SMA-like *Smn*$^{2B/-}$ littermates in presymptomatic, early and late symptomatic stages. Indeed, these findings were counter to our hypothesis, which we based on numerous previous studies that report alterations in skeletal muscle protein levels between control and SMA-like animals[5,31,34,35,48]. Nonetheless, the present results suggest that important molecular machinery critical for driving exercise responses is maintained in the SMA-like condition and that their exercise induction would presumably therefore be preserved. Despite the maintenance of AMPK and p38, expression of their downstream target PGC-1α was attenuated in SMA-like mice compared to their healthy littermates. Incongruences such as these between AMPK and PGC-1α levels or p38 and PGC-1α have been noted

previously[26]. The depressed PGC-1α expression observed here are consistent with reports of lower PGC-1α gene expression and impaired mitochondrial respiration in the skeletal muscle of SMA patients[49–51] and may be attributed, in part, to impaired myogenesis previously reported in the SMA condition[34,49].

A single bout of exercise evokes a rise in AMPK and p38 activity in healthy animals and humans[17]. Furthermore, acute physical activity also drives the myonuclear translocation and activation of their downstream target PGC-1α[52]. Within the nuclear compartment, the coactivator positively influences its transcription via an autoregulatory loop[53]. Our data demonstrate, for the first time, exercise-induced stimulation of the AMPK-p38-PGC-1α signaling axis in the skeletal muscle of SMA-like animals. Specifically, a single bout of physical activity was sufficient to augment AMPK and p38 activation status, PGC-1α mRNA, as well as its myonuclear accumulation. Interestingly, the activation of the upstream kinases AMPK and p38 occurred immediately after exercise and preceded the cellular translocation and transcription of PGC-1α, which was detected 3 hours post. These results confirm our earlier assertion that the maintenance of AMPK and p38 expression at healthy levels in *Smn*$^{2B/-}$ mice is permissive for their induction in response to exercise. This is also reflected in the exercise-evoked myonuclear translocation and transcription of PGC-1α, which recapitulates results previously observed in the healthy condition[52]. It is reasonable to posit, therefore, that chronic activation of AMPK-p38-PGC-1α signaling underlies many of the adaptations associated with exercise training in SMA[28]. Indeed, chronic pharmacological activation of these kinases mitigate the severity in pre-clinical models of the most prevalent NMDs of children and adults, including Duchenne muscular dystrophy (DMD), myotonic dystrophy type 1, and SMA[28,40]. Whether additive,

or synergistic, effects of physical activity combined with pharmacological treatments emerge warrants investigation.

Recent work from Frédéric Charbonnier's laboratory strongly suggests that the AKT-CREB and ERK-ELK pathways regulate SMN expression in skeletal muscle in a reciprocal manner[4,5]. More specifically, AKT-CREB signaling promotes SMN transcription while the ERK-ELK cascade represses it. With this in mind, we sought to investigate the effects of physical activity on the upstream regulation of SMN transcription. While exercise evoked rapid, robust, but transient AKT activation in the muscle of SMA-like mice, CREB signaling was unaffected. This result is not without precedent however, as unaltered CREB activity in skeletal muscle in response to acute exercise has been previously observed[54]. It is important to note here that this finding does not preclude the possibility that other molecules downstream of AKT were stimulated by physical activity in *Smn*$^{2B/-}$ animals. Examining the effect of exercise on alternative targets of AKT such as p53 for example[55], is worth pursuing particularly since inhibition of p53 has been shown prevent cell death in SMA[56]. Consistent with previous work[4,5], we found that ERK-ELK signaling was elevated in the muscles of SMA-like mice compared to their healthy counterparts. This upregulation under basal conditions likely contributes to mechanisms suppressing SMN expression in SMA[5,40,57]. Thus, relief from the repressive effects of the ERK-ELK cascade might trigger increased SMN transcription and raise the abundance of full-length SMN. This idea is supported by the increased SMN expression that occurred coincident with attenuated ERK-ELK signaling in the muscle of SMA-like mice that were deficient in the insulin growth factor-1 receptor[5]. Along these lines, our results demonstrate that a single bout of exercise in *Smn*$^{2B/-}$ mice was able to correct skeletal muscle ERK-ELK signaling. This normalization, or supercompensation in the case of ELK activation status,

ensued rapidly, however whether the duration of this acute physical activity-evoked amelioration extends beyond 3 hours post-exercise is unknown and deserves further investigation.

Our data demonstrate positive exercise-induced effects on the AMPK-p38-PGC-1α axis, as well as on AKT and the ERK-ELK cascade in the skeletal muscle of SMA-like mice. A logical extension of these results would be to then examine whether these alterations were associated with enhanced full-length SMN expression. As expected, a single bout of physical activity did not affect SMN protein content. In contrast however, we provide the first evidence that acute exercise was successful at eliciting significant elevations in full-length SMN transcript levels. Collectively, the present results provide strong support for our earlier speculation[28,40], that the mechanism(s) responsible for exercise-induced SMN expression may include enhanced SMN transcriptional activation downstream of AKT and ERK/ELK signaling[5], PGC-1α-driven pre-mRNA splicing corrections[58,59], as well as p38-mediated SMN mRNA stabilization[10,47]. Furthermore, the data demonstrate that physiological AMPK stimulation is associated with increased SMN expression, which extends an earlier proof-of-concept report that observed some benefits to pharmacological AMPK activation in severe SMA-like mice[26]. Previous studies present conflicting results regarding the effects of chronic exercise on SMN expression in SMA-like mice[5,27,44], which are likely due, at least in part, to the disparate SMA murine models utilized and training variables selected. Nevertheless, this important pre-clinical work reveals that significant cellular and physiological benefits, including prolonged lifespan, are likely gleaned by both SMN-dependent and -independent mechanisms. These seminal studies, complemented by the current results, underscore the necessity to continue

searching for optimal exercise conditions, for example, frequency, intensity, duration and mode, which will evoke the most robust benefits in SMA.

A single bout of exercise was sufficient to stimulate the master regulator of autophagy ULK1 and normalize some indicators of aberrant autophagic signaling, such as ULK1 and BNIP3 gene expression, as well as p62 content and LC3 ratios, in the muscles of *Smn*$^{2B/-}$ mice. Dysregulated autophagy has been previously reported in these animals[9], and pharmacological or genetic correction of autophagic pathways provides favorable outcomes in SMA-like mice[8,9]. The activation of autophagy is critical for meditating exercise training-induced adaptations in healthy animals[60], as well as in alternative pre-clinical models of the compromised neuromuscular system such as advanced aging for instance[61] and in DMD[62]. As skeletal muscle ULK1 activation is dependent on functional AMPK and since exercise-induced remodeling requires ULK1[63], it is reasonable to postulate that the augmented activation status of AMPK and ULK1 evoked by acute physical activity in SMA-like mice that were observed in the present study are important for precipitating downstream events that facilitate structural and functional improvements in SMA brought about by exercise training[28]. As such, further investigation of the therapeutic potential of targeting the AMPK-ULK1 axis in SMA is justified.

In summary, our data shows that AMPK and p38, potent remodelers of neuromuscular phenotype, retain critical expression and activation characteristics in the skeletal muscle of pre-clinical SMA-like *Smn*$^{2B/-}$ mice. We also observed in these animals that exercise-induced AMPK-p38-PGC-1α activation, coincident with AKT- and ERK/ELK-mediated mechanisms, were associated with enhanced full-length SMN expression. A single bout of physical activity also stimulated the AMPK-ULK1 axis and resulted in some measure of corrected autophagic signaling. These acute events, although

alone insufficient to cause stable, adaptive changes, are likely necessary and therefore represent essential upstream molecular and cellular components of the beneficial effects of chronic exercise training observed in the SMA context[28]. Current understanding of SMA places a tremendous emphasis on the importance of improved peripheral tissue health, skeletal muscle in particular, to delaying the presentation and progression of the disorder[64–66], through cell-autonomous mechanisms, as well as by cell non-autonomous processes via retrograde influence of muscle on their αMNs. Thus, continued examination of skeletal muscle biology in SMA, as well as the mechanisms of exercise adaptation in pre-clinical models and SMA patients, may lead to novel, effective therapeutic strategies to mitigate this disorder.

References

1. Prior, T. W. *et al.* Newborn and carrier screening for spinal muscular atrophy. *Am. J. Med. Genet. Part A* **152,** 1608–1616 (2010).
2. Ogino, S., Leonard, D. G. B., Rennert, H., Ewens, W. J. & Wilson, R. B. Genetic risk assessment in carrier testing for spinal muscular atrophy. *Am. J. Med. Genet.* **110,** 301–307 (2002).
3. Lefebvre, S. *et al.* Correlation between severity and SMN protein level in spinal muscular atrophy. *Nat. Genet.* **16,** 265–269 (1997).
4. Biondi, O. *et al.* Exercise-Induced Activation of NMDA Receptor Promotes Motor Unit Development and Survival in a Type 2 Spinal Muscular Atrophy Model Mouse. *J. Neurosci.* **28,** 953–962 (2008).
5. Biondi, O. *et al.* IGF-1R Reduction Triggers Neuroprotective Signaling Pathways in Spinal Muscular Atrophy Mice. *J. Neurosci.* **35,** 12063–12079 (2015).
6. Han, K.-J. *et al.* Ubiquitin-specific protease 9x deubiquitinates and stabilizes the spinal muscular atrophy protein-survival motor neuron. *J. Biol. Chem.* **287,** 43741–52 (2012).
7. Kwon, D. Y. *et al.* The E3 ubiquitin ligase mind bomb 1 ubiquitinates and promotes the degradation of survival of motor neuron protein. *Mol. Biol. Cell* **24,** 1863–1871 (2013).
8. Rodriguez-Muela, N. *et al.* Blocking p62/SQSTM1-dependent SMN degradation ameliorates Spinal Muscular Atrophy disease phenotypes. *J. Clin. Invest.* (2018). doi:10.1172/JCI95231
9. Deguise, M.-O. *et al.* Differential induction of muscle atrophy pathways in two mouse models of spinal muscular atrophy. *Sci. Rep.* **6,** 28846 (2016).

10. Farooq, F. *et al.* Celecoxib increases SMN and survival in a severe spinal muscular atrophy mouse model via p38 pathway activation. *Hum. Mol. Genet.* **22,** 3415–3424 (2013).

11. d'Ydewalle, C. *et al.* The Antisense Transcript SMN-AS1 Regulates SMN Expression and Is a Novel Therapeutic Target for Spinal Muscular Atrophy. *Neuron* **93,** 66–79 (2017).

12. Powis, R. A. *et al.* Systemic restoration of UBA1 ameliorates disease in spinal muscular atrophy. *JCI insight* **1,** e87908 (2016).

13. Passini, M. a *et al.* Antisense oligonucleotides delivered to the mouse CNS ameliorate symptoms of severe spinal muscular atrophy. *Sci. Transl. Med.* **3,** 72ra18 (2011).

14. Hua, Y. *et al.* Motor neuron cell-nonautonomous rescue of spinal muscular atrophy phenotypes in mild and severe transgenic mouse models. *Genes Dev.* **29,** 288–97 (2015).

15. Hua, Y. *et al.* Peripheral SMN restoration is essential for long-term rescue of a severe spinal muscular atrophy mouse model. *Nature* **478,** (2011).

16. Hawley, J. A., Hargreaves, M., Joyner, M. J. & Zierath, J. R. Integrative biology of exercise. *Cell* **159,** 738–749 (2014).

17. Egan, B. & Zierath, J. R. Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell Metabolism* **17,** 162–184 (2013).

18. Gardiner, P. F. Changes in alpha-motoneuron properties with altered physical activity levels. *Exerc. Sport Sci. Rev.* **34,** 54–8 (2006).

19. Desaulniers, P. *et al.* Habitual exercise enhances neuromuscular transmission efficacy of rat soleus muscle in situ Habitual exercise enhances neuromuscular

transmission efficacy of rat soleus muscle in situ. *J. Appl. Physiol.* **90,** 1041–1048 (2001).

20. Arnold, A. S. *et al.* Morphological and functional remodelling of the neuromuscular junction by skeletal muscle PGC-1a. *Nat. Commun.* **5,** (2014).
21. Jasmin, B. J., Lavoie, P.-A. & Gardiner, P. F. Fast axonal transport of acetylcholinesterase in rat sciatic motoneurons is enhanced following prolonged daily running, but not following swimming. *Neurosci. Lett.* **78,** 156–172 (1987).
22. Jasmin, B. J., Lavoie, P. A. & Gardiner, P. F. Fast axonal transport of labeled proteins in motoneurons of exercise-trained rats. *Am. J. Physiol.* **255,** C731–C736 (1988).
23. Andonian, M. & Fahim*, M. Endurance Exercise Alters the Morphology of Fast- and Slow-Twitch Rat Neuromuscular Junctions. *Int. J. Sports Med.* **09,** 218–223 (1988).
24. Deschenes, M. R. *et al.* The effects of exercise training of different intensities on neuromuscular junction morphology. *J. Neurocytol.* **22,** 603–615 (1993).
25. Kang, C. M., Lavoie, P. A. & Gardiner, P. F. Chronic exercise increases SNAP-25 abundance in fast-transported proteins of rat motoneurones. *Neuroreport* **6,** 549–553 (1995).
26. Cerveró, C. *et al.* Chronic Treatment with the AMPK Agonist AICAR Prevents Skeletal Muscle Pathology but Fails to Improve Clinical Outcome in a Mouse Model of Severe Spinal Muscular Atrophy. *Neurotherapeutics* **13,** 198–216 (2016).
27. Grondard, C. *et al.* Regular Exercise Prolongs Survival in a Type 2 Spinal. *J. Neurosci.* **25,** 7615–7622 (2005).
28. Ng, S. Y., Manta, A. & Ljubicic, V. Exercise biology of neuromuscular disorders.

Appl. Physiol. Nutr. Metab. 1–44 (2018).

29. Charbonnier, F. Exercise-induced neuroprotection in SMA model mice: A means for determining new therapeutic strategies. *Molecular Neurobiology* **35,** 217–223 (2007).

30. Egan, B. & Zierath, J. R. Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell Metabolism* **17,** 162–184 (2013).

31. Monani, U. R. *et al.* The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in Smn(-/-) mice and results in a mouse with spinal muscular atrophy. *Hum. Mol. Genet.* **9,** 333–339 (2000).

32. Osborne, M. *et al.* Characterization of behavioral and neuromuscular junction phenotypes in a novel allelic series of SMA mouse models. *Hum. Mol. Genet.* **21,** 4431–4447 (2012).

33. Bowerman, M., Murray, L. M., Beauvais, A., Pinheiro, B. & Kothary, R. A critical smn threshold in mice dictates onset of an intermediate spinal muscular atrophy phenotype associated with a distinct neuromuscular junction pathology. *Neuromuscul. Disord.* **22,** 263–276 (2012).

34. Boyer, J. G. *et al.* Myogenic program dysregulation is contributory to disease pathogenesis in spinal muscular atrophy. *Hum. Mol. Genet.* **23,** 4249–4259 (2014).

35. Boyer, J. G. *et al.* Early onset muscle weakness and disruption of muscle proteins in mouse models of spinal muscular atrophy. *Skelet. Muscle* **3,** 24 (2013).

36. Liu, H., Yazdani, A., Murray, L. M., Beauvais, A. & Kothary, R. The Smn-independent beneficial effects of trichostatin a on an intermediate mouse model of spinal muscular atrophy. *PLoS One* **9,** 3–11 (2014).

37. Hood, D. A. Mechanisms of exercise-induced mitochondrial biogenesis in skeletal

muscle. *Appl. Physiol. Nutr. Metab.* **472,** 465–472 (2009).

38. Granata, C., Jamnick, N. A. & Bishop, D. J. Principles of Exercise Prescription, and How They Influence Exercise-Induced Changes of Transcription Factors and Other Regulators of Mitochondrial Biogenesis. *Sports Medicine* **48,** 1541–1559 (2018).
39. MacInnis, M. J. & Gibala, M. J. Physiological adaptations to interval training and the role of exercise intensity. *Journal of Physiology* **595,** 2915–2930 (2017).
40. Dial, A. G., Ng, S. Y., Manta, A. & Ljubicic, V. The Role of AMPK in Neuromuscular Biology and Disease. *Trends Endocrinol. Metab.* **29,** 300–312 (2018).
41. Schmittgen, T. D. & Livak, K. J. Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc.* **3,** 1101–1108 (2008).
42. Ljubicic, V., Burt, M. & Jasmin, B. J. The therapeutic potential of skeletal muscle plasticity in Duchenne muscular dystrophy: Phenotypic modifiers as pharmacologic targets. *FASEB J.* **28,** 548–568 (2014).
43. Millino, C. *et al.* Different atrophy-hypertrophy transcription pathways in muscles affected by severe and mild spinal muscular atrophy. *BMC Med.* **7,** 14 (2009).
44. Chali, F. *et al.* Long-term exercise-specific neuroprotection in spinal muscular atrophy-like mice. *J. Physiol.* **594,** 1931–1952 (2016).
45. Vainshtein, A., Tryon, L. D., Pauly, M. & Hood, D. A. Role of PGC-1α during acute exercise-induced autophagy and mitophagy in skeletal muscle. *Am. J. Physiol. - Cell Physiol.* **308,** C710–C719 (2015).
46. Coffey, V. G. & Hawley, J. A. The molecular bases of training adaptation. *Sports Medicine* **37,** 737–763 (2007).
47. Farooq, F., Balabanian, S., Liu, X., Holcik, M. & MacKenzie, A. p38 Mitogen-

activated protein kinase stabilizes SMN mRNA through RNA binding protein HuR. *Hum. Mol. Genet.* **18,** 4035–4045 (2009).

48. Lee, Y. Il, Mikesh, M., Smith, I., Rimer, M. & Thompson, W. Muscles in a mouse model of spinal muscular atrophy show profound defects in neuromuscular development even in the absence of failure in neuromuscular transmission or loss of motor neurons. (2011). doi:10.1016/j.ydbio.2011.05.667
49. Ripolone, M. *et al.* Impaired Muscle Mitochondrial Biogenesis and Myogenesis in Spinal Muscular Atrophy. *JAMA Neurol.* **72,** 1–10 (2015).
50. Berger, A. *et al.* Severe depletion of mitochondrial DNA in spinal muscular atrophy. *Acta Neuropathol.* **105,** 245–51 (2003).
51. Sperl, W. *et al.* High resolution respirometry of permeabilized skeletal muscle fibers in the diagnosis of neuromuscular disorders. *Mol. Cell. Biochem.* **174,** 71–78 (1997).
52. Wright, D. C. *et al.* Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1a expression. *J. Biol. Chem.* **282,** 194–199 (2007).
53. Handschin, C. *et al.* PGC-1α regulates the neuromuscular junction program and ameliorates Duchenne muscular dystrophy. *Genes Dev.* **21,** 770–783 (2007).
54. Widegren, U., Wretman, C., Lionikas, A., Hedin, G. & Henriksson, J. Influence of exercise intensity on ERK/MAP kinase signalling in human skeletal muscle. *Pflugers Arch. Eur. J. Physiol.* (2000). doi:10.1007/s004240000417
55. Gottlieb, T. M., Leal, J. F. M., Seger, R., Taya, Y. & Oren, M. Cross-talk between Akt, p53 and Mdm2: possible implications for the regulation of apoptosis. *Oncogene* **21,** 1299–1303 (2002).
56. Simon, C. M. *et al.* Converging Mechanisms of p53 Activation Drive Motor Neuron Degeneration in Spinal Muscular Atrophy. *Cell Rep.* **21,** 3767–3780 (2017).

57. Ahmad, S., Bhatia, K., Kannan, A. & Gangwani, L. Molecular mechanisms of neurodegeneration in spinal muscular atrophy. *J. Exp. Neurosci.* **10,** 39–49 (2016).

58. Monsalve, M. *et al.* Direct coupling of transcription and mRNA processing through the thermogenic coactivator PGC-1. *Mol. Cell* **6,** 307–316 (2000).

59. Martínez-Redondo, V. *et al.* Peroxisome proliferator-activated receptor γ coactivator-1 α isoforms selectively regulate multiple splicing events on target genes. *J. Biol. Chem.* **291,** 15169–15184 (2016).

60. Lira, V. A. *et al.* Autophagy is required for exercise training-induced skeletal muscle adaptation and improvement of physical performance. *FASEB J.* **27,** 4184–4193 (2013).

61. Hood, D. A., Memme, J. M., Oliveira, A. N. & Triolo, M. Maintenance of Skeletal Muscle Mitochondria in Health, Exercise, and Aging. *Annu. Rev. Physiol.* **81,** (2019).

62. Hulmi, J. J. *et al.* Exercise restores decreased physical activity levels and increases markers of autophagy and oxidative capacity in myostatin/activin-blocked mdx mice. *AJP Endocrinol. Metab.* **305,** E171–E182 (2013).

63. Laker, R. C. *et al.* Ampk phosphorylation of Ulk1 is required for targeting of mitochondria to lysosomes in exercise-induced mitophagy. *Nat. Commun.* **8,** 548 (2017).

64. Bowerman, M. *et al.* Therapeutic strategies for spinal muscular atrophy: SMN and beyond. *Dis. Model. Mech.* **10,** 943–954 (2017).

65. Groen, E. J. N., Talbot, K. & Gillingwater, T. H. Advances in therapy for spinal muscular atrophy: promises and challenges. *Nat. Rev. Neurol.* (2018). doi:10.1038/nrneurol.2018.4

66. Wood, H. Motor neuron disease: Benefits of nusinersen extend to later-onset SMA. *Nat. Publ. Gr.* **14,** (2018).