Effects of long-term voluntary exercise and aging in rats on markers of Long Interspersed Nuclear Element-1 (L1) activity in skeletal muscle, liver, and brain tissue

By

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ABSTRACT

Long Interspersed Nuclear Element-1 (LINE-1 or L1) is the only active mammalian TE. L1 is an autonomous retrotransposon that is able to “copy and paste” itself, increasing its presence as it does so and leading to genomic instability. L1 has been implicated in multiple diseases and the aging process. Our laboratory has shown L1 expression and regulation can be favorably altered through acute exercise, highlighting another aspect of the importance of exercise in maintaining health and cell integrity. We sought to determine the effects of chronic, voluntary wheel running on L1 expression and regulation, as well as markers of the cGAS-STING inflammatory pathway. Female Lewis rats (n=34) were separated into one of three groups. A group analyzed at 6 months old to serve as a young comparator group (CTL, n=10). From there, two groups were aged out to 15 months old. One group had access to a running wheel for voluntary wheel running from 6 months to 15 months of age (EX, n=12), while the other group did not and served as a sedentary control from 6 months to 15 months of age (SED, n=12). Plantaris, soleus, liver, and hippocampus tissues were harvested, and RNA, DNA, and protein were isolated for analysis. Methylation of the L1 promoter was significantly higher in SED and EX compared to CTL for both L1-3 and L1-Tot ($p=0.021$ and $p=0.028$, respectively) in the soleus and hippocampus tissues. ORF1p expression was higher in older SED and EX rats compared to CTL for every tissue. There were no differences between groups in protein expression for the cGAS-STING pathway markers. Our results suggest there is an increased ORF1p protein expression across tissues with aging that is not mitigated by voluntary wheel running. Additionally, while previous data imply that L1 methylation changes

play may a role in acute exercise for L1 RNA expression, this does not seem to occur during extended periods of voluntary wheel running.

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CHAPTER I

INTRODUCTION

Long Interspersed Nuclear Element-1 (LINE-1 or L1) is a class 1 autonomous retrotransposon that can "copy and paste" itself through an RNA intermediate [1]. Because of this mechanism of retrotransposition, L1 accounts for 17% of human DNA [2], 23% of rat DNA [3], and 18% of mouse DNA [4]. Notably, though L1 makes up a large percentage of mammalian DNA, not all these copies are able to actively retrotranspose in the genome [5]. In human cells, it has been estimated that there are roughly 100 active copies of this gene [6], where there are 500 active copies in rats [3] and 3,000 in mice [7].

A full-length L1 that has maintained the ability to undergo retrotransposition is 6 kilobases in length and contains a 5' untranslated region (UTR) with an internal promoter, two open reading frames (ORF1 and ORF2), and a 3' UTR [8] [5]. Active copies of this gene are transcribed in full, and the resultant transcript is bicistronic meaning that it encodes for two proteins (ORF1p and ORF2p). Once these proteins are translated from the L1 transcript, both bind with *cis* preference to the L1 transcript resulting in L1 ribonucleoprotein particles (L1-RNP). Critically, ORF1p binds in trimers to an available L1 transcript to aid in translocation of the mRNA back into the nucleus [9, 10]. Once in the nucleus, ORF2p possesses endonuclease and reverse transcriptase activities that nick the DNA and prime for reverse transcription, respectively [11, 12]. This process is referred to as target-primed reverse transcription (TPRT) and occurs pseudo-randomly, leaving the possibility of interrupting protein-coding genes and adding to genomic instability. It is currently

thought that cleavage occurs in a consensus 5'-TTTT/A-3' motif, where "/" indicates the cleavage site [11, 13].

Multiple studies suggest the L1-RNP needs a break in the nuclear envelope to return to the nucleus to undergo retrotransposition [14, 15]. Thus, this process can occur in actively dividing cells. However, given that muscle cells are post-mitotic and do not undergo cellular division to disrupt the nuclear envelope, it is questionable as to whether this process occurs in myofibers. Notwithstanding, Gorbunova and colleagues have shown that an accumulation of L1 cDNA copies in the cytoplasm can induce an inflammatory response through the cGAS DNA sensing pathway [16]. Therefore, L1-RNPs that accumulate in the sarcoplasm may induce this type I interferon response, and this may be an involved mechanism of skeletal muscle dysfunction, particularly with aging.

The nature of retrotransposition has various implications in both diseases and aging, so the regulation of L1 activity has been established within the cell to stave off these effects [17-19]. Regulation of expression is accomplished largely through epigenetic mechanisms such as DNA methylation and chromatin alterations [20-22]. In the context of disease, L1 activity is not always the driving factor, but it has been estimated the L1 TPRT accounts for 1 in every 250 pathogenic mutations [17, 18]. Hemophilia, Duchenne's muscular dystrophy, rheumatoid arthritis, and cancer are diseases that have been well documented to occur via L1-driven mutagenesis [19]. With the establishment of advanced sequencing, scientists have determined where the de novo insertions of L1 can affect gene expression in tumor tissues [23, 24]. Notably, chromatin rearrangements and methylation status are fundamental biological processes that are altered in cancer [25, 26]. Aging

is defined as a failure or decline in the ability of cells, tissues, and organ systems to maintain homeostasis and proper function to cope with environmental stressors [27]. Changes in methylation status and chromatin structure are well-characterized in the aging phenomenon [28, 29]. Studies regarding these concepts led to the idea that aging leads to altered epigenetic regulation of retrotransposons [30-32].

It has been well established that exercise is a potent stimulus for positive skeletal muscle adaptation. In fact, significant molecular adaptations can occur with as little as one bout of exercise [33], and several studies have shown that one or multiple bouts of exercise can alter the epigenetic landscape in older individuals [34-37]. Previous data from our laboratory have shown that exercise may have a role in regulating L1 expression in skeletal muscle [38-40]. Particularly, we have shown the effects of both resistance and endurance exercise in rodent and human models. There is a consensus among these studies that L1 mRNA decreases with exercise, and these alterations seem to be due to alterations in L1 methylation at the internal promoter site. However, our data are limited to an acute exercise bout in humans [40], as well as shorter-tern training in humans and rats [38, 39]. Moreover, all of these data are limited to skeletal muscle analysis.

Given the data presented above along with previous data collected from our laboratory, the goal of the present study is to better characterize the effect of chronic exercise on the regulation of L1 expression in a rodent model. Previous studies from our laboratory have focused on mixed fiber skeletal muscles (i.e. gastrocnemius and vastus lateralis), so we aimed to examine plantaris and soleus muscles to determine any effects of fiber type on markers of L1 activity. Any differences in activity or regulation could lead to a better understanding of mechanisms of action in the future.

Additionally, we sought to determine how long-term exercise in rats affected markers of L1 activity in the liver and the hippocampus given that both of these tissues positively adapt to exercise stimuli [41].

CHAPTER II

REVIEW OF LITERATURE

Discovery of TEs and Classification

Transposable elements (TEs) were discovered in the 1950s and are special pieces of DNA that are mobile throughout the genome [42-44]. Barbara McClintock pioneered the discovery of these elements with a phenomenon first observed in maize, where two genetic loci were able to change positions (or transpose) within a chromosome and cause a mutation. It was discovered that this genetic event ultimately led to different colors being expressed by the maize kernels. Briefly, McClintock noted a unique interaction within the Ac/Ds loci positioned on chromosome 9 whereby the Ds, or dissociation locus, can move around the genome when activated by the Ac, or activator locus. The Ac locus is considered an autonomous "mutable locus", and Ds requires Ac for mutability, leading to these different phenotypes. This was discovered by crossing maize plants until the Ac locus was absent. In this case, the Ds locus was unable to transpose. This finding led to her theory that these mobile elements could regulate gene expression, ultimately spawning the notion that the genome is a dynamic, not static, structure. Historically, TEs were considered "junk DNA" and largely ignored for decades. Since this discovery, multiple studies have shown the important role TEs have in cell biology, gene regulation, and health and disease [17, 45, 46]. TEs make up a large portion of mammalian genomes, accounting for ~50% of the human genome [2] and 38% of the mouse genome [4].

As this field of research grew, a classification system was proposed in 1989 by Finnegan to distinguish TEs based on their transposition intermediate [47]. This led to the division into two

classes: Class I (retrotransposons) and Class II (DNA transposons). Class I TEs use an RNA intermediate for transposition, and function in a 'copy and paste' mechanism [48], while Class II TEs use DNA as an intermediate and function in a 'cut and paste' mechanism [1]. Since this original classification, further discoveries have warranted other classification systems to further delineate differences between TEs. Wicker et al. set out to solve this issue by using a hierarchical approach to include more levels within the two classes which distinguished between the various mechanistic and enzymatic differences between TEs [1]. Class I TEs, specifically, are the focus of this review and can be further divided into autonomous and nonautonomous elements. Autonomous retrotransposons include endogenous retroviruses (ERVs) and Long Interspersed Nuclear Elements (LINEs), while nonautonomous retrotransposons include Short Interspersed Nuclear Elements (SINEs) and SINE-VTNR-ALUs (SVAs). The LINE subfamily, particularly LINE-1 (or L1 as written herein), is considered the most active mammalian retrotransposon. The high level of L1 activity and association with various diseases has made it a prime candidate for biomedical research [24, 49, 50].

Long Interspersed Nuclear Element-1 Biology

L1 is a class I autonomous retrotransposon that can "copy and paste" itself through an RNA intermediate [1]. It is considered autonomous because the L1 gene encodes for the proteins necessary to undergo retrotransposition and does not require other protein interactions to do so [1]. L1 accounts for 17% of human DNA [2], 23% of rat DNA [3], and 18% of mouse DNA [4]. Notably, though L1 makes up a large percentage of mammalian DNA, not all copies are actively

able to retrotranspose in the genome [5]. In humans, there are roughly 100 active copies of this gene [6], where there are 500 active copies in rats [3] and 3,000 in mice [7].

A full-length L1 that has maintained the ability to undergo retrotransposition is 6 kilobases in length and contains a 5' untranslated region, two open reading frames (ORF1 and ORF2) that are translated into the ORF1p and ORF2p proteins, and a 3' untranslated region [5, 8]. The 5' UTR contains an intact internal promoter that allows for recruitment of RNA polymerase II to facilitate transcription. The 3' UTR contains a polyadenylated tail that can be used to distinguish the younger more active LINEs from the older inactive ones [8]. Active copies of this gene are transcribed in full, and the resultant transcript is bicistronic meaning that it encodes for two proteins (ORF1p and ORF2p). Once these proteins are translated from the L1 transcript, both bind with *cis* preference to the L1 transcript resulting in L1 ribonucleoprotein particles (L1-RNP). ORF1p is 40 kDa in size and acts as an RNA binding protein, preferentially binding in trimers to L1 mRNA transcripts [9, 10]. ORF2p is a 150 kDa protein that contains both endonuclease and reverse transcriptase activities that are essential for integrating new L1 copies into the genome (i.e., *de novo* L1 retrotransposition) [11, 12].

In order for retrotransposition to occur, the gene first has to be transcribed by RNA polymerase II in the nucleus. Mature L1 transcripts are transported out of the nucleus to the ribosomes in the cytoplasm where the mRNA is translated into the respective ORF1p and ORF2p proteins. Thereafter, the proteins assemble a ribonucleoprotein particle (RNP) in a cis-preferential fashion by binding to an accessible L1 transcript [51]. ORF1p binds in multiple trimers along the transcript and may serve to protect the transcript while aiding in chaperoning the RNP back into

the nucleus. ORF2p binds near the 3' end of the transcript and uses its multiple activities to integrate the L1 copy into the genome once the RNP has relocated to the nucleus. ORF2p has both endonuclease (EN) and reverse transcriptase (RT) activities, where EN is used to nick the DNA and create a free 3' OH group that the RT activity can use as a primer to integrate the transcript into the DNA. It is currently accepted that the EN cleavage from ORF2p occurs at a consensus 5'-TTTT/A-3' motif, where "/" indicates the cleavage site [11, 13].

It has been posited that the L1 RNP requires a break in the nuclear envelope to return to the nucleus to undergo retrotransposition [14, 15]. Given that muscle cells are post-mitotic and do not undergo cellular division to disrupt the nuclear envelope, it is debatable as to whether L1 retrotransposition occurs in myofibers. Notwithstanding, L1 accumulation in the cytoplasm can have adverse consequences. For instance, Gorbunova and colleagues have shown that an accumulation of L1 cDNA copies in the cytoplasm can induce an inflammatory response through the cGAS DNA sensing pathway [16]. Bai and Liu go into great detail regarding the cGAS pathway and its implications [52]. Briefly, the cGAS-cGAMP-STING signaling cascade begins with the sensing and binding of dsDNA to cGAS to activate it. Once activated, cGAS catalyzes the formation of 2'3'-cGAMP, which then activates STING proteins. Subsequently, STING is able to translocate and recruit TBK1, setting off a cascade that results in type I interferon (IFN) production. Thus, L1-RNPs that accumulate in the sarcoplasm may induce this type I interferon response, and this may be an involved mechanism regarding the adverse effects that L1 has on skeletal muscle.

Regulation of L1 Expression

L1 has been shown to negatively impact the genome in multiple instances, so cellular mechanisms have been developed to silence these TEs. Well-established L1 repressive mechanisms include increasing DNA methylation at the L1 promoter or chromatin alterations leading to the confinement of L1 in heterochromatic DNA regions [20-22].

DNA methylation is an epigenetic modification that leads to changes in gene expression through the addition or removal of methyl groups to cytosine nucleotides to form 5-methylcytosine [53]. Methylation of gene promoters is accomplished through the deposition of methyl groups on CpG islands, and increased methylation is generally associated with the repression of gene expression [54, 55]. By keeping L1 expression under control through hypermethylation, the cell can maintain genome stability and proper function. The 5' UTR of L1 contains approximately 13% CpG islands, and the promoter can exhibit a methylation state of 20-100% [56, 57]. In general, DNA methyltransferases DNMT3A, DNMT3B, DNMT3L are responsible for *de novo* methylation, targeting CpG islands in hemi-and unmethylated contexts [58, 59]. Additionally, DNMT1, a canonical maintenance DNA methyltransferase, also has retrotransposition specific targeting of *de novo* methylation, while it is not as prevalent as DNMT3A [59].

L1 gene expression can also be modulated through changes in chromatin structure and, more specifically, through histone modifications. Chromatin is dynamic in its ability to tighten or loosen in order to repress or enhance gene expression, respectively. Histones can be methylated and acetylated through histone methyltransferases and acetyltransferases, respectively, to alter TE expression and the expression of other genes through the promotion of a heterochromatic or

euchromatic environment [60-62]. When a promoter is in a euchromatic environment, transcriptional machinery has access to the gene and can promote its expression. Conversely, a heterochromatic environment leads to repression due to a lack of access to the promoter region. Histone 3 lysine 9 (H3K9) is an example of a histone mark that is tightly regulated through these alterations to control retrotransposon activity. Trimethylation of H3K9 (H3K9me3) through histone methyltransferases leads to heterochromatin formation and ultimately gene silencing [60, 63, 64]. H3K9 can also be acetylated (H3K9ac) and is associated with increased gene expression through transcription initiation and the formation of a euchromatic environment [65]. Histone acetylation is a reversible process led by histone deacetylase (HDAC) enzymes [66]. Specifically, it has been reported that L1 is regulated by histone deacetylation, and this is largely catalyzed by NAD^+ dependent HDAC, SIRT6 [67]. SIRT6 can bind to the 5'UTR of L1 where it facilitates the packing of L1 elements into a heterochromatic state. In the instance of aging and DNA damage, SIRT6 is depleted from L1 promoters, allowing for its activation [67].

While not as heavily researched, there are other means of repressing retrotransposons in eukaryotes. RNA binding proteins have been shown to modulate L1 activity through alterations in mRNA stability or ORF protein activity. APOBEC3 is a gene family encoding for proteins that catalyze the deamination of cytidine to uridine [68]. APOBEC3A and 3B can inhibit L1 through deaminase pathways that target L1 strands during TPRT, reducing the activity of ORF1p [69-71]. The Moloney leukemia virus 10 (MOV10), an RNA helicase protein part of the RNA-induced silencing complex (RISC), has been shown to interact with the L1 RNP to promote L1 mRNA

degradation in cell culture [72]. TREX1, a cytoplasmic DNA exonuclease, has been shown to reduce ORF1p levels and reduce L1-RNP-mediated nicking of DNA in cell culture [73].

Implications of L1 – Disease and Aging

Due to the nature of TPRT and random insertion at a consensus motif, L1 has been implicated in various diseases. In this regard, it has been estimated the L1 TPRT accounts for 1 in every 250 pathogenic mutations [17, 18], ranging from hemophilia, Duchenne's muscular dystrophy, rheumatoid arthritis, and cancer [19].

The first report of the deleterious effects L1 can have on the genome was from Kazazian and colleagues, where they examined over 200 boys with hemophilia A and discovered two of them had truncated mutagenic L1 insertions within the gene coding region of coagulation factor VIII [74]. After examining the mother of one of the subjects, they discovered a full-length L1 copy, identical to the truncated insertion found in her son [75]. There is also a notable L1 presence in tumorigenesis, as ORF1p expression is considered a hallmark of high-grade malignant cancers [76]. The first account of such a contribution was in a colorectal cancer patient where a L1 insertion disrupted the coding exon of APC, a tumor suppressor gene [77]. With the establishment of advanced sequencing, scientists have shown numerous instances where L1 insertions affect gene expression in tumor tissues [23, 24]. Notably, chromatin rearrangements and methylation status are fundamental biological processes that are altered in cancer [25, 26]. To this extent, L1 hypomethylation has been reported in virtually all human cancers and recognized as a prognostic marker for both disease stage and disease-free survival prediction [26, 78, 79].

Aging is defined as a failure or decline in the ability of cells, tissues, and organ systems to maintain homeostasis and cope with environmental stressors [27]. In regards to skeletal muscle, aging is associated with genomic instability and epigenetic alterations, leading to cellular senescence and atrophy [80]. L1 expression has been hypothesized to be involved in this aging phenomenon due to circumstances previously discussed. L1 mRNA expression and integration into DNA have been shown to increase with age in multiple studies [30, 31, 81].

Retrotransposition has the ability to alter the genomic landscape, and there is also the opportunity to insert directly into a protein coding region, rendering the gene nonfunctional. Methylation and chromatin changes are hallmarks of both aging and L1 expression. Particularly, global DNA hypomethylation is seen with biological aging, and it has been posited that these alterations are important to the lifespan [29, 82]. As previously mentioned, methylation is an important regulator of L1 expression, whereas hypermethylation leads to decreased L1 expression. Global hypomethylation, therefore, provides a mechanism where L1 expression is able to increase. Chromatin changes that occur with aging include rearrangements through euchromatin formation [83, 84]. Certain histone marks that repress gene expression through heterochromatin formation change with age, altering gene expression [85, 86]. This alteration in chromatin structure led to the idea that aging leads to altered epigenetic regulation of retrotransposons [28]. A study by DeCecco and colleagues supported this idea by demonstrating these chromatin changes led to an increase in transcription and TPRT of L1 [31].

Given that increased L1 expression increases the potential for pathogenicity, failure to maintain methylation and chromatin structure during aging can be troublesome. DeCecco and

colleagues have shown this possibility in both liver and skeletal muscle tissues [30]. Specifically, the authors observed an increase in L1 DNA copy number in both tissues in 36-month-old mice compared to both 5- and 24-month-old mice. Moreover, the authors reported a relaxation of heterochromatic regions of DNA and speculated this change contributed to the increased transcription of L1. Furthermore, our laboratory published a study in rats with similar findings [32]. Specifically, gastrocnemius muscle from 3-, 12-, and 24-month-old rats were analyzed for L1 markers, and L1 mRNA expression was higher in the 12- and 24-month rats. Additionally, L1 DNA content, ORF1p levels, and L1 DNA content in euchromatic regions were all higher in 24- versus 3-month-old rats. Together, these findings warrant research into how L1 retrotransposition during aging affects the homeostasis of various tissues and organ systems within the body.

Exercise and L1

It has been well established that exercise is a potent stimulus for adaptation [33]. These adaptations span multiple systems including skeletal muscle, cardiorespiratory, and neuromuscular systems [87-90]. Specific to previously discussed mechanisms of regulation, exercise has been shown to affect the epigenetic status of skeletal muscle [34-36]. These alterations allow for better adaptations in future bouts of exercise, yielding what has been termed muscle "memory" [34, 35]. Previous data from our laboratory has shown that exercise may have a role in regulating L1 expression in skeletal muscle. Particularly, we have shown the effects of both resistance and endurance exercise in rodent and human models. Romero et al. was the first report where both acute and chronic resistance exercise in college-aged males resulted in a significant decrease in

vastus lateralis skeletal muscle L1 activity, largely through increases in methylation of the L1 promoter [38]. Similarly, we published a rodent study where rats selectively bred for high voluntary wheel running were given a running wheel for 22 weeks [39]. Compared to a sedentary age- and strain-matched control group, gastrocnemius L1 mRNA expression was significantly lower and there was higher L1 promoter methylation in the exercise group. Lastly, a human study from our laboratory examined the effects of aging and exercise on L1 activity [40]. With respect to aging, ORF1 mRNA was higher and DNA methylation was lower in vastus lateralis muscle of older humans. Interestingly, however, one bout of exercise was able to transiently downregulate ORF1 mRNA levels, regardless of age.

Purpose of this study

Given the data presented in this review along with data collected from our laboratory, the goal of the present study is to better characterize the effect of chronic endurance exercise on the regulation of L1 expression in a rodent model. Specifically, we want to further characterize these effects in multiple tissues with longer term exercise than previously performed. To do this, we will examine L1 mRNA expression, ORF1p expression, and protein expression related to the cGAS-STING DNA sensing pathway. Furthermore, we will characterize the regulation of L1 expression through promoter methylation status and mRNA expression of genes that regulate L1 expression. Our hypotheses are that, while aging will result in higher L1 expression across tissues, exercise will reduce L1 expression to a more youth-like status. Additionally, we hypothesize that exercise training will increase L1 promoter methylation. To test these hypotheses we will analyze liver,

hippocampus, and the soleus and plantaris muscles from 6-month-old, 15-month-old sedentary, and 15-month-old rodents that underwent 9 months of daily voluntary wheel running. This analysis of multiple tissues will provide a more comprehensive view of the effect that exercise has on markers of L1 activity. Additionally, examining plantaris and soleus skeletal muscle will allow us to determine any effects of fiber type on L1 activity.

CHAPTER III

METHODS

Animals

Prior to the commencement of the study, all experimental procedures were approved by Auburn University's Institutional Animal Care and Use Committee (IACUC; protocol no. 2020-3647). Three month (mo) old female Lewis rats were purchased (Envigo, Indianapolis, IN) and kept in the animal housing facility prior to experimentation. During acclimation, animals were provided water and fed a standard rodent chow (24% protein, 58% carbohydrate, 18% fat; Harlan Laboratories) ad libitum in a maintained ambient temperature and constant 12:12-h light-dark cycle.

Experimental Protocol

At 6 months of age, animals were separated into one of three groups. The first group was euthanized at 6 months (CTL; n = 10) to serve as a young comparator group. The two remaining groups were placed in either a standard rodent cage without a running wheel and considered a sedentary group (SED; n = 12) or a cage with a running wheel and allowed to run voluntarily on a daily basis (EX; n = 12). All animals were singly housed with a nylabone and fed standard chow ad libitum for the duration of the study. Data was collected weekly to track body masses and food intakes in both groups, as well as running distances and time spent on running wheels for the EX group. Additionally, running wheels were locked 20 hours before EX animals were euthanized to avoid acute exercise-induced signaling occurrences from being detected.

Necropsies

On the morning of necropsies, rats were food-deprived for >4 hours and transported from the campus vivarium to the School of Kinesiology where they acclimated for 3 hours. Thereafter, rats were euthanized under CO_2 gas in a 2-liter induction chamber (VetEquip, Pleasanton, CA) and cervical separation was performed as a secondary measure. Following euthanasia, a final body mass was recorded, and blood was collected from the heart using a 22-gauge syringe. Collected blood was placed in a 6-ml serum separator tube (SST). SST blood was centrifuged at 3,500 g for 10 min, and resultant serum was then aliquoted into 1.7-ml microcentrifuge tubes and stored at -80°C for further analysis. The right-side inguinal/subcutaneous adipose tissue (SQ), right side perirenal adipose tissue, right-side triceps brachii, right-side gastrocnemius, right-side soleus, and right-side plantaris, whole liver, and bilateral hippocampus were dissected out and weighed using a calibrated scale with a sensitivity of 0.0001 g (Mettler-Toledo; Columbus, OH). Following dissection and weight procurement, tissues were wrapped in a labeled foil, flash-frozen in liquid nitrogen, and stored in the -80°C for downstream analyses.

Protein Isolation and Western Blotting

Tissues were removed from -80°C storage and crushed on a liquid nitrogen-cooled ceramic mortar and pestle. Approximately 20 mg of each respective tissue was placed in 1.7 microcentrifuge tubes containing 500 µL of ice-cold cell lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na-EDTA, 1 mM EGTA, 1% Triton] (Cell Signaling; Danvers, MA, USA). Tissues were homogenized using tight-fitting pestles and then centrifuged at 500 g for 5 min. Supernatants were transferred to new 1.7 mL tubes, and protein concentrations were determined using a BCA assay (Thermo Scientific, Waltham, MA, USA).

Lysates were prepared for SDS-PAGE using 4x Laemmli buffer at 1 μg/μL for Western blot analysis, and 15 μL were loaded onto 4-15% SDS-polyacrylamide pre-casted gels (Bio-Rad Laboratories; Hercules, CA, USA). 1x SDS-PAGE run buffer (VWR Laboratories; Randor, PA, USA) was used for electrophoresis at 180 V for 60 min. Thereafter, proteins were transferred via 200 mA constant current for 120 min to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were then stained with Ponceau S and imaged using a gel documentation system (ChemiDoc Touch, Bio-Rad) to ensure equal loading of samples among lanes. Membranes were subsequently blocked at room temperature with 5% nonfat milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST) for one hour. All of the following primary antibodies were incubated overnight at 4°C in a solution of TBST containing 5% bovine serum albumin (BSA; Ameresco): mouse anti-total OXPHOS rodent (1:1000; Abcam, cat# ab110413), rabbit anti-cGAS (1:1000; Cell Signaling, cat# 15102), rabbit anti-STING (1:1000, Cell Signaling, cat# 50494), rabbit anti-phospho-STING (Ser365) (1:1000, Cell Signaling, cat# 72971), rabbit anti-HNE (1:1000; Cell Biolabs, San Diego, CA, cat# STA-035). Additionally, ORF1p antibodies were procured from Myeloma P3X63-Ag8.653 cell culture supernatants (1:20, cells graciously provided by Dr. Gerald Schumann; more information can be found in the reference [91]). The following day, membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (1:2000; Cell Signaling, cat# 7076 and 7074, respectively) in a solution of TBST with 5% BSA for one hour. Thereafter, membranes were developed using Luminata Forte HRP substrate (EMD Millipore, Billerica, MA, USA). Images were taken and band density values were assessed with the ChemiDoc Touch gel documentation system and associated densitometry software. All

densitometry values for proteins of interest were divided by Ponceau densitometry values. These values were then normalized to the group mean of CTL to yield relative protein expression levels.

RNA Isolation and cDNA Synthesis for qPCR Analysis

Tissues were removed from -80ºC storage and crushed on a liquid nitrogen-cooled ceramic mortar and pestle. Approximately 10 mg of muscle was used to isolate RNA via the Ribozol method per the manufacturer's recommendations (VWR International). Following RNA isolation, the RNA pellet was reconstituted in 30 µl of RNase-free water, and RNA concentrations were determined in duplicate at an absorbance of 260 nm by using a NanoDrop Lite (Thermo Scientific, Waltham, MA, USA). A 1% agarose gel was used to check for isolated RNA for DNA contamination. Thereafter, cDNA (2 µg) was synthesized using a commercial qScriptTM cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) per the manufacturer's recommendations.

qPCR was performed with gene-specific primers and SYBR-green-based methods (Quanta Biosciences) in a real-time PCR thermal cycler (Bio-Rad). The final volume of qPCR reactions was 20 µl, which contained a final concentration of 2 µM of forward and reverse primers and 25 ng of cDNA. All reactions were performed in duplicate. Primers were designed in our lab to interrogate L1 mRNA expression based on previous data from Kirilyuk et al. [91]. Briefly, both primers amplified a portion of the 5' UTR, while L1-3 was designed to probe for the most active L1 element and L1-Tot was designed to probe for all full-length L1 elements, most of which are not able to undergo retrotransposition. Forward and reverse sequences for all genes are located in Table 1. Fold-change values from the CTL group were performed using the $2^{\Delta\Delta Cq}$ method where:

$2^{\Delta Cq}$ = 2^[housekeeping gene (HKG) Cq – gene of interest Cq]

and,

$2^{\Delta\Delta Cq}$ (or fold-change) = [$2^{\Delta Cq}$ value/$2^{\Delta Cq}$ average of CTL group].

Fibrillarin (*Fbl*) was used as the housekeeping gene for plantaris and soleus, while cyclophilin A (*Ppia*) was used for liver and hippocampus.

Table 1. Rat primer sequences for qPCR

Gene	Accession number
(Rat) Fibrillarin (*Fbl*; HKG) FP (5'→3') CTGCGGAATGGAGGACACTT RP (5'→3') GATGCAAACACAGCCTCTGC	NM_001025643.1
(Rat) Cyclophilin A (*Ppia*; HKG) FP (5'→3') GCATACAGGTCCTGGCATCT RP (5'→3') AGCCACTCAGTCTTGGCAGT	NM_012583.2
(Rat) L1 (L1-3) FP (5'→3') GACCATCTGGAACCCTGGTG RP (5'→3') GGGCCTGTGTCTTGAGTTCA	DQ100473.1
(Rat) L1 (L1-Tot) FP (5'→3') GGAAGAGACCACCAACACTG RP (5'→3') GAAGGTTTAGCTCTCCCTCC	DQ100473.1 DQ100475.1 DQ100476.1 DQ100477.1 DQ100474.1 DQ100482.1
(Rat) (Mov10) FP (5'→3') TCAGCAAACAGCACAGATCC RP (5'→3') AGACAAACTGGGGTGTCCAG	NM_001107711.2
(Rat) (Trex1) FP (5'→3') CTGCCCTGAAGACCTTGGAG RP (5'→3') GGCTTCCACTGACAGATGCT	NM_001024989.1
(Rat) (Apobec3D) FP (5'→3') CTCTGTGGCGATCAGGGATC RP (5'→3') ATCTTACAGGCCCCAGGACT	NM_001033703.2

Legend: Forward and reverse primer sequences used for qPCR. HKG, housekeeping gene

DNA Isolation

Approximately 10 mg of frozen tissue was processed using the commercially available DNeasy Blood & Tissue Kit (QIAGEN, Venlo, Netherlands) per the manufacturer's recommendations including RNase treatment. Following DNA precipitation and pelleting, DNA was eluted with 100 μL of elution buffer from the kit per manufacturer's recommendations, and DNA concentrations were determined in duplicate at an absorbance of 260 nm by using a NanoDrop Lite (Thermo Scientific, Waltham, MA, USA).

DNA Methylation of the L1 Promoter

L1 promoter methylation analysis was performed on isolated DNA using a commercially available methylated DNA immunoprecipitation (MeDIP) kit (Abcam, Cambridge, MA, USA). Prior to performing the MeDIP assay, 1.5 μg of DNA was digested using MseI (New England BioLabs, Ipswich, MA, USA). Following digestion reactions, 1 μg of DNA was used for immunoprecipitation with an anti-5- methylcytosine antibody provided within the kit. qPCR was then performed on the methylated DNA enriched sample using the L1 promoter primer sets. Additionally, 0.1 μg of residual input DNA from each sample was used as a control in a parallel reaction to normalize qPCR results. Both the experimental and control wells contained 25 ng of DNA for the reactions and were carried out using the same primer- and SYBR green-based methods as described above for qPCR. Fold-change scores in L1 DNA methylation were calculated as follows:

a) 2ΔCq values were calculated whereby ΔCq = input DNA Cq – methylated DNA Cq, and

b) fold-change values were then obtained by dividing each individual 2ΔCq value by the CTL 2ΔCq group mean.

Citrate Synthase Activity

Citrate synthase activity assays were done for plantaris, soleus, liver, and hippocampus tissues. The assay principle is based upon the reduction of 5,50-dithiobis (2- nitrobenzoic acid) (DTNB) at 412 nm (extinction coefficient 13.6 mmol/L/cm) coupled to the reduction of acetyl-CoA by the citrate synthase reaction in the presence of oxaloacetate. Samples were isolated according to the protein isolation protocol above and prepped at 1 μg/μL before being added to a mixture composed of 0.125 mol/L Tris–HCl (pH 8.0), 0.03 mmol/L acetyl-CoA, and 0.1 mmol/L DTNB. The reaction was initiated by the addition of 5 μL of 50 mmol/L oxaloacetate and the absorbance change was recorded for 1 min.

Statistics

Statistics were performed using SPSS v 23.0 (IBM, Armonk, NY, USA). Shapiro-Wilk testing was used to determine normality for all dependent variables prior to statistical analysis. In cases where data were not normally distributed, Kruskal-Wallis (K-W) tests were used to compare dependent variables between groups, with Mann-Whitney U post hoc tests were used to detect where significance was present. For normally distributed data, one-way ANOVAs were used with LSD post hoc tests. Variables collected weekly were analyzed using independent samples t-tests.

Statistical significance was established at $p \leq 0.05$ for all null hypothesis testing. Data herein are presented as mean ± standard deviation values.

CHAPTER IV

COMPLETED MANUSCRIPT (to be submitted to Journal of Applied Physiology in Sp 2022)

Long-term voluntary wheel running and aging effects on markers of Long Interspersed Nuclear Element-1 in skeletal muscle, liver, and brain tissue of female rats

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ABSTRACT

We sought to determine the long-term voluntary wheel running and aging effects on markers of Long Interspersed Nuclear Element-1 (L1) in skeletal muscle, liver, and brain tissue of female rats. Additionally, markers of the cGAS-STING inflammatory pathway were interrogated. Female Lewis rats (n=34) were separated into one of three groups including a 6-month-old group to serve as a young comparator group (CTL, n=10), a group that had access to a running wheel for voluntary wheel running from 6-15 months old (EX, n=12), and an age-matched group that did not (SED, n=12). There were no significant differences in L1 mRNA expression for any of the tissues between groups. Methylation of the L1 promoter in the soleus and hippocampus was significantly higher in SED and EX compared to CTL ($p<0.05$). ORF1p expression was higher in older SED and EX rats compared to CTL for every tissue ($p<0.05$). There were no differences between groups for L1 mRNA or cGAS-STING pathway markers. Our results suggest there is an increased ORF1p protein expression across tissues with aging that is not mitigated by voluntary wheel running. Additionally, while previous data imply that L1 methylation changes play may a role in acute exercise for L1 RNA expression, this does not seem to occur during extended periods of voluntary wheel running.

Keywords: L1, cGAS-STING, aging, exercise

INTRODUCTION

Long Interspersed Nuclear Element-1 (LINE-1 or L1) is a class 1 autonomous retrotransposon that can "copy and paste" itself through an RNA intermediate [1]. L1 accounts for 17% of human DNA [2], 23% of rat DNA [3], and 18% of mouse DNA [4]. Notably, although L1 makes up a large percentage of mammalian DNA, not all these copies are capable of active retrotransposition [5]. In human cells, it has been estimated that there are roughly 100 retrotransposition-competent copies of this gene [6], where there are 500 copies in rats [3] and 3,000 in mice [7].

A full-length L1 that has maintained the ability to undergo retrotransposition is 6 kilobases in length and contains a 5' untranslated region (UTR) with an internal promoter, two open reading frames (ORF1 and ORF2), and a 3' UTR [5, 8]. Active copies of this gene are transcribed in full, and the resultant transcript encodes for two proteins, ORF1p and ORF2p. After translation, the proteins bind with *cis* preference to the L1 transcript in a specific manner, resulting in L1 ribonucleoprotein particles (L1-RNP). During retrotransposition, ORF1p binds in trimers to an available L1 transcript to aid in translocation of the mRNA back into the nucleus [9, 10]. Once in the nucleus, ORF2p, bound to the 3' end of the transcript, possesses endonuclease and reverse transcriptase activities that nick the DNA and prime for reverse transcription, respectively [11, 12]. This process is referred to as target-primed reverse transcription (TPRT) and it is currently thought that cleavage occurs in a consensus 5'-TTTT/A-3' motif, where "/" indicates the cleavage site [11, 13]. Furthermore, because TPRT occurs pseudo-randomly, the

possibility of interrupting protein-coding genes is a considerable threat to maintaining genomic stability, especially as the organism ages.

Recent studies suggest the L1-RNP needs a break in the nuclear envelope to return to the nucleus to undergo retrotransposition [14, 15]. While this process can occur in actively dividing cells, post-mitotic cells (such as myofibers) do not undergo cellular division. Thus, it is questionable as to whether this process occurs in myofibers. Interestingly, Gorbunova and colleagues have shown that the progeroid *Sirt6*-knockout mouse model shows heightened L1 mRNA expression, and this causes an accumulation of cytoplasmic L1 cDNA in various tissues [16]. High levels of L1 cDNA in these tissues subsequently activated the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway, which in turn, drove the expression of pro-inflammatory type I interferons. Similar results have been reported in human cell lines as well [17]; specifically, the overexpression of an engineered L1 construct has been shown to activate interferon signaling. Given previous literature suggesting that muscle aging is largely associated with inflammatory processes [18-20], the idea that cytoplasmic L1 cDNA accumulation contributes to age-associated muscle inflammation is a compelling proposition.

L1 markers have been examined in several diseases, and the regulation of L1 expression has been well-established [17-19]. Regulation is accomplished largely through epigenetic mechanisms such as DNA methylation and chromatin alterations [20-22]. In the context of disease, it has been estimated the L1 TPRT accounts for one in every 250 pathogenic mutations [17, 18]. Hemophilia, Duchenne's muscular dystrophy, rheumatoid arthritis, and certain cancers are diseases that have been documented to occur via L1-driven mutagenesis [19]. With the

establishment of advanced sequencing, scientists have determined where the *de novo* insertions of L1 can affect gene expression in tumor-bearing tissues [23, 24]. Aging, on the other hand, is largely defined as a failure or decline in the ability of cells, tissues, and organ systems to maintain homeostasis and proper function to cope with environmental stressors [27]. Additionally, changes in methylation status and chromatin structure are well-characterized in the aging phenomenon [28, 29]. Studies in this area have begun examining the epigenetic regulation of retrotransposons [30-32]. Outside of epigenetic regulation, there are various RNA binding proteins that are associated with the repression of L1 activity. Specifically, APOBEC3 [70], MOV10 [72], and TREX1 [73] are all able to modulate L1 activity by altering mRNA stability or ORF protein activity.

It has been well established that exercise stimulates positive skeletal muscle adaptations. In fact, significant molecular adaptations can occur with as little as one bout of exercise [33], and several studies have shown that one or multiple bouts of exercise can alter the epigenetic landscape in older individuals [34-37]. Our laboratory has shown that exercise may have a role in regulating L1 expression in skeletal muscle [38-40]. These studies indicated that L1 mRNA decreases with various forms of exercise, and these alterations seem to be due to alterations in L1 methylation at the internal promoter site. However, our data are limited to an acute resistance exercise bout in younger adult men [40], as well as shorter-term endurance training in older humans and younger female rats [38, 39]. Further, all these studies only provided data on skeletal muscle. Given the data presented above along with previous data collected from our laboratory, the goal of the present study was to better characterize the effects of chronic voluntary wheel

running and aging on the L1 markers in female rats. Previous studies from our laboratory have focused on mixed fiber skeletal muscles (i.e., gastrocnemius and vastus lateralis), so we aimed to examine plantaris (>90% type II fibers) and soleus (>90% type I fibers) muscles to determine any effects of fiber type on markers of L1 activity. Additionally, we examined L1 markers in the liver and the hippocampus given that both of these tissues have been researched regarding L1 expression [30, 92, 93], and both tissues positively adapt to exercise stimuli [41]. Finally, cGAS-STING markers were examined in all tissues of interest given the link between L1 and the cGAS-STING pathway.

MATERIALS AND METHODS

Animals

Prior to the commencement of the study, all experimental procedures were approved by Auburn University's Institutional Animal Care and Use Committee (IACUC; protocol no. 2020-3647). Three-month (mo)-old female Lewis rats were purchased from a commercial vendor (Envigo, Indianapolis, IN) and kept in the animal housing facility prior to experimentation. During acclimation, animals were provided water and fed a standard rodent chow (24% protein, 58% carbohydrate, 18% fat; Harlan Laboratories) ad libitum in a maintained ambient temperature and constant 12:12-h light-dark cycle.

Experimental Protocol

At 6 months of age, animals were separated into one of three groups. The first group was euthanized at 6 months (CTL; n = 10) to serve as a young comparator group. The two remaining groups were placed in either a standard rodent cage without a running wheel and considered a sedentary group (SED; n = 12) or a cage with a running wheel and allowed to run voluntarily daily (EX; n = 12). All animals were singly housed with a nylabone and fed standard chow ad libitum for the duration of the study. Data was collected weekly to track body masses and food intakes in both groups, as well as running distances and time spent on running wheels for the EX group. Additionally, running wheels were locked 20 hours before EX animals were euthanized to avoid acute exercise-induced signaling occurrences from being detected.

Necropsies

On the morning of necropsies, rats were food-deprived for >4 hours and transported from the campus vivarium to the School of Kinesiology where they acclimated for 3 hours. Thereafter, rats were euthanized under CO_2 gas in a 2-liter induction chamber (VetEquip, Pleasanton, CA) and cervical separation was performed as a secondary measure. Following euthanasia, a final body mass was recorded. The right-side inguinal/subcutaneous adipose tissue (SQ), right side perirenal adipose tissue, right-side triceps brachii, right-side gastrocnemius, right-side soleus, and right-side plantaris, whole liver, and bilateral hippocampus were dissected out and weighed using a calibrated scale with a sensitivity of 0.0001 g (Mettler-Toledo; Columbus, OH). Following dissection and weight procurement, tissues were wrapped in a labeled foil, flash-frozen in liquid nitrogen, and stored in the -80°C for downstream analyses.

Protein Isolation and Western Blotting

Tissues were removed from -80°C storage and crushed on a liquid nitrogen-cooled ceramic mortar and pestle. Approximately 20 mg of each respective tissue was placed in 1.7 microcentrifuge tubes containing 500 µL of ice-cold cell lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na-EDTA, 1 mM EGTA, 1% Triton] (Cell Signaling; Danvers, MA, USA). Tissues were homogenized using tight-fitting pestles and then centrifuged at 500 g for 5 min. Supernatants were transferred to new 1.7 mL tubes, and protein concentrations were determined using a BCA assay (Thermo Scientific, Waltham, MA, USA).

Lysates were prepared for SDS-PAGE using 4x Laemmli buffer at 1 µg/µL for Western blot analysis, and 15 µL were loaded onto 4-15% SDS-polyacrylamide pre-casted gels (Bio-Rad Laboratories; Hercules, CA, USA). 1x SDS-PAGE run buffer (VWR Laboratories; Randor, PA, USA) was used for electrophoresis at 180 V for 60 min. Thereafter, proteins were transferred via 200 mA constant current for 120 min to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were then stained with Ponceau S and imaged using a gel documentation system (ChemiDoc Touch, Bio-Rad) to ensure equal loading of samples among lanes. Membranes were subsequently blocked at room temperature with 5% nonfat milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST) for one hour. The following primary antibodies were incubated overnight at 4°C in a solution of TBST containing 5% bovine serum albumin (BSA; Ameresco): mouse anti-total OXPHOS rodent (1:1000; Abcam, cat# ab110413), rabbit anti-cGAS (1:1000; Cell Signaling, cat# 15102), rabbit anti-STING (1:1000, Cell

Signaling, cat# 50494), rabbit anti-phospho-STING (Ser365) (1:1000, Cell Signaling, cat# 72971). Additionally, ORF1p antibodies were procured from Myeloma P3X63-Ag8.653 cell culture supernatants (1:10; cells graciously provided by Dr. Gerald Schumann; more information can be found in the reference [91]). The following day, membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (1:2000; Cell Signaling, cat# 7076 and 7074, respectively) in a solution of TBST with 5% BSA for one hour. Thereafter, membranes were developed using Luminata Forte HRP substrate (EMD Millipore, Billerica, MA, USA). Images were taken and band density values were assessed with the ChemiDoc Touch gel documentation system (Bio-Rad, Hercules, CA, USA) and associated densitometry software. All densitometry values for proteins of interest were divided by Ponceau densitometry values, except p-STING densitometry values being divided by pan-STING values.

RNA Isolation and cDNA Synthesis for qPCR Analysis

Tissues were removed from -80ºC storage and crushed on a liquid nitrogen-cooled ceramic mortar and pestle. Approximately 10 mg of muscle was used to isolate RNA via the Ribozol method per the manufacturer's recommendations (VWR International). Following RNA isolation, the RNA pellet was reconstituted in 30 µl of RNase-free water, and RNA concentrations were determined in duplicate at an absorbance of 260 nm by using a NanoDrop Lite (Thermo Scientific, Waltham, MA, USA). A 1% agarose gel was used to check for isolated RNA for DNA contamination. Thereafter, cDNA (2 µg) was synthesized using a commercial

qScriptTM cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) per the manufacturer's recommendations.

qPCR was performed with gene-specific primers and SYBR-green-based methods (Quanta Biosciences) in a real-time PCR thermal cycler (Bio-Rad). The final volume of qPCR reactions was 20 µl, which contained a final concentration of 2 µM of forward and reverse primers and 25 ng of cDNA. All reactions were performed in duplicate. Primers were designed in our lab [39] to interrogate L1 mRNA expression based on previous data from Kirilyuk et al. [91]. Briefly, both primers amplified a portion of the 5' UTR, while L1-3 was designed to probe for the most active L1 element and L1-Tot was designed to probe for all full-length L1 elements, most of which are not able to undergo retrotransposition. Forward and reverse sequences for all genes are in Table 1. Fold-change values from the CTL comparator group were performed using the $2^{\Delta\Delta Cq}$ method where $2^{\Delta Cq}$ = 2^[housekeeping gene (HKG) Cq – gene of interest Cq], and $2^{\Delta\Delta Cq}$ (or fold-change) = [$2^{\Delta Cq}$ value/$2^{\Delta Cq}$ average of CTL group]. Fibrillarin (*Fbl*) was used as the housekeeping gene for plantaris and soleus, while cyclophilin A (*Ppia*) was used for liver and hippocampus.

INSERT TABLE 1 HERE

DNA Isolation

Approximately 10 mg of frozen tissue was processed using the commercially available DNeasy Blood & Tissue Kit (QIAGEN, Venlo, Netherlands) per the manufacturer's

recommendations including RNase treatment. Following DNA precipitation and pelleting, DNA was eluted with 100 μL of elution buffer from the kit per manufacturer's recommendations, and DNA concentrations were determined in duplicate at an absorbance of 260 nm by using a NanoDrop Lite (Thermo Scientific, Waltham, MA, USA).

DNA Methylation of the L1 Promoter

L1 promoter methylation analysis was performed on isolated DNA using a commercially available methylated DNA immunoprecipitation (MeDIP) kit (Abcam, Cambridge, MA, USA). Prior to performing the MeDIP assay, 1.5 μg of DNA was digested using MseI (New England BioLabs, Ipswich, MA, USA). Following digestion reactions, 1 μg of DNA was used for immunoprecipitation with an anti-5- methylcytosine antibody provided within the kit. qPCR was then performed on the methylated DNA enriched sample using the L1 promoter primer sets. Additionally, 0.1 μg of residual input DNA from each sample was used as a control in a parallel reaction to normalize qPCR results. Both the experimental and control wells contained 25 ng of DNA for the reactions and were carried out using the same primer- and SYBR green-based methods as described above for qPCR. Fold-change scores in L1 DNA methylation were calculated as follows:

a) $2^{\Delta Cq}$ values were calculated whereby ΔCq = input DNA Cq – methylated DNA Cq, and

b) fold-change values were then obtained by dividing each individual $2^{\Delta Cq}$ value by the CTL $2^{\Delta Cq}$ group mean.

Citrate Synthase Activity

Citrate synthase activity assays were performed for plantaris, soleus, liver, and hippocampus tissues. The assay principle is based upon the reduction of 5,50-dithiobis (2-nitrobenzoic acid) (DTNB) at 412 nm (extinction coefficient 13.6 mmol/L/cm) coupled to the reduction of acetyl-CoA by the citrate synthase reaction in the presence of oxaloacetate. Samples were isolated according to the protein isolation protocol above and prepped at 1 μg/μL before being added to a mixture composed of 0.125 mol/L Tris–HCl (pH 8.0), 0.03 mmol/L acetyl-CoA, and 0.1 mmol/L DTNB. The reaction was initiated by the addition of 5 μL of 50 mmol/L oxaloacetate and the absorbance change was recorded for 1 min.

Statistics

Statistics were performed using SPSS v 23.0 (IBM, Armonk, NY, USA). Shapiro-Wilk testing was used to determine normality for all dependent variables prior to statistical analysis. In cases where data were not normally distributed, Kruskal-Wallis (K-W) tests were used to compare dependent variables between groups, with Mann-Whitney U post hoc tests were used to detect where significance was present. For normally distributed data, one-way ANOVAs were used with LSD post hoc tests. Variables collected weekly were analyzed using independent samples t-tests. Statistical significance was established at $p \leq 0.05$ for all null hypothesis testing. Data herein are presented as mean ± standard deviation values.

RESULTS

Phenotype and Exercise Effects

Throughout the duration of the study, run distance, food intake, and body mass were measured weekly. Average run distance was tracked in the exercise group to determine the endpoint of the study (Figure 1a). It was determined *a priori* that the study would conclude when the exercise group average running distance fell below 20 km a week for two consecutive weeks, so exercise adaptations were not lost. As a result, the study lasted 36 weeks, and EX rats ran for 9 total months. Body mass was tracked weekly (Figure 1d). There was no difference between groups at the beginning of the study ($p=0.267$), but from week 10 through week 36, EX weighed significantly more than SED ($p<0.05$). Weekly food intake was also measured, and total food consumed was used to determine the average feed efficiency (total grams body mass gained/total kcal consumed) over the duration of the study (Figure 1b and 1c). EX consumed significantly more food compared to SED from week 4 through the end of the study ($P<0.05$), but there were no differences in total feed efficiency between groups ($p=0.096$).

INSERT FIGURE 1 HERE

Tissue weights were collected at the time of necropsy to determine absolute and relative tissue mass as shown in Figures 2a and 2b, respectively. Notably, there were significant differences in liver absolute (CTL<SED<EX) and relative masses (CTL>EX>SED) (ANOVA $p<0.001$). Brain absolute mass was significantly different between all groups (CTL<SED<EX) (K-W $p=0.001$) and there was an age effect on the relative mass, where the SED and EX were less than CTL (ANOVA $p<0.001$). Plantaris absolute mass was significantly different between

all groups (CTL<SED<EX) (ANOVA $p<0.001$) and relative mass was significantly higher in CTL compared to SED and EX (K-W $p<0.001$). Soleus absolute tissue mass was higher in EX compared to CTL and SED (K-W $p<0.001$) while relative mass was different between all groups (CTL<EX<SED).

INSERT FIGURE 2 HERE

Citrate synthase activity (Figure 3a) and total OXPHOS complex band densities determined using western blotting (Figures 3b-3e) were analyzed to characterize any exercise adaptations. Citrate synthase activity was not significantly different between groups in all tissues. Conversely, there were differences in OXPHOS complexes within the skeletal muscles. Specifically, complex I was significantly different in the plantaris, where CTL was higher than SED and EX (K-W $p=0.036$) (Figure 3b). Complexes I, II, and III were significantly different in the soleus. Complex I was higher in EX compared to CTL (K-W $p=0.032$), complex II was higher in EX compared to CTL and SED (K-W $p=0.011$), and complex III was higher in EX compared to SED (ANOVA $p=0.012$).

INSERT FIGURE 3 HERE

L1 DNA methylation and mRNA expression

We initially sought to focus on L1 DNA methylation because of its high impact on L1 regulation. Due to low and inconsistent signals in the plantaris and liver samples, only the soleus and hippocampus are presented for the methylation status of the L1 internal promoter.

Methylation was higher in SED and EX compared to CTL for soleus L1-3 and L1-Tot (K-W p=0.021 and p=0.028, respectively; Figure 4a). Methylation was higher in EX compared to CTL and SED for hippocampus L1-3 and L1-Tot (K-W p=0.002 and p=0.005, respectively; Figure 4b).

INSERT FIGURE 4 HERE

L1 mRNA expression based on L1-3 and L1-Tot primers is characterized in Figure 5. While there were no significant differences in L1 mRNA in any tissues analyzed, there were trends. Specifically, soleus L1-3 (K-W p=0.082; Figure 5b), soleus L1-Tot (K-W p=0.059; Figure 5b), and hippocampus L1-3 (K-W p=0.072; Figure 5d) trended where EX was higher than CTL in each instance.

INSERT FIGURE 5 HERE

ORF1p protein expression and the mRNA expression of select L1 inhibitors

Despite no differences in L1 mRNA expression, there was an effect of aging on ORF1p expression in the tissues analyzed (Figure 6). SED and EX were higher than CTL for plantaris (ANOVA p=0.028), soleus (K-W p=0.001), and liver (K-W p=0.036) tissues. Notably, hippocampus ORF1p was so lowly expressed, we could not quantify it (*data not presented*).

INSERT FIGURE 6 HERE

Another form of L1 regulation has been addressed with RNA binding proteins. Of note, we wanted to investigate how the RNA transcripts of TREX1, APOBEC3, and MOV10 were expressed within each group (Figure 7). Soleus MOV10 mRNA and liver APOBEC3 mRNA were significantly higher in SED and EX compared to CTL (K-W p=0.011 and p=0.004, respectively).

INSERT FIGURE 7 HERE

cGAS-STING markers

Given that the cGAS-STING DNA sensing pathway has been implicated as being involved with detecting heightened levels of L1 in various tissues, we examined whether proteins associated with this inflammatory pathway were altered in any of the groups. Liver cGAS protein was greater in SED and EX compared to CTL (ANOVA p=0.043), but this marker was not different in other assayed tissues. Additionally, there was no difference between groups in any tissue for phospho-/pan STING expression.

INSERT FIGURE 8 HERE

DISCUSSION

While we have previously examined the effects of exercise on L1 activity in skeletal muscle, the effects of a longer-term exercise paradigm on L1 markers across various tissues have not been characterized to date. Of note, we observed increased ORF1p expression with aging that chronic voluntary wheel running was not able to mitigate, and this was evident regardless of the

nonsignificant differences in L1 mRNA expression that existed between groups. Additionally, there was an increase in methylation of the L1 promoter in soleus and hippocampus tissues with aging, and long-term voluntary wheel running seemingly affect this marker. Finally, aside from heightened liver cGAS expression in the EX and SED versus CTL group, there was no effect of aging or long-term voluntary wheel running on markers of the cGAS-STING pathway in other tissues. We contend that most of our null findings related to exercise may have been due to the voluntary wheel running stimulus not causing appreciable adaptations as evidenced through the CS activity and mitochondrial complex protein data across all tissues. However, certain age-related findings are interesting and are in line with some of our previous data. Both topics will be discussed in greater detail below.

We did not observe any significant differences in L1 mRNA expression between groups. This contrasts previous research given that DeCecco and colleagues observed an increase in L1 expression in mice aged 24 and 36 months old compared to 5 months [30]. Additionally, our laboratory observed heightened L1 mRNA expression in 12- and 24-month-old rats compared to 3-month-old rats [32]. We posit that SED and EX rats in the current study, being 15 months old, may not have been old enough to present increased L1 mRNA expression when considering that these effects were seen in much older rodents by De Cecco and colleagues. Indeed, Mumford and colleagues showed that 12-month-old rats presented higher L1 mRNA levels relative to 3-month-old rats. However, this was observed in the mixed fiber gastrocnemius muscle, versus the soleus and plantaris muscles that were examined herein, and the younger CTL rats were 6 months old (versus 3-month-old rats analyzed by Mumford and colleagues). Hence, age

differences as well as the examination of different muscles between all the aforementioned studies likely explain discrepant findings.

In terms of exercise effects on L1 mRNA, Romero and colleagues have reported that ~4 months of voluntary wheel running in rats significantly decreased L1 mRNA expression in the gastrocnemius muscle [39]. Again, there are possible explanations for the discrepancies in the current study compared to this previous finding by our laboratory. First, Romero and colleagues examined rats that were bred for high running distances, where the rats we used were standard laboratory rats. Additionally, the rats analyzed by Romero and colleagues spanned from 5 weeks to 27 weeks of age. It is likely that these rats were transitioning from pubertal phases to adulthood, so the difference in phase of life could result in divergent findings [94]. Regarding past human findings, we have shown an increase in muscle L1 mRNA expression in older (58 ± 8 years old) participants [40], and a decrease in L1 mRNA expression with three consecutive resistance exercise in college-aged participants [38]. However, Romero et al. [38] reported that chronic resistance exercise (12 weeks) did not significantly decrease L1 mRNA expression. This collective evidence supports the notion that one exercise bout, versus chronic exercise training, may not elicit the same L1 mRNA expression response. Moreover, there may be differences in exercise modality in terms of the stimulus provided to affect L1 mRNA expression.

Despite no differences being observed between groups in L1 mRNA expression, ORF1p protein expression was shown to be greater in the older EX and SED groups compared to CTL rats, and this was not mitigated with chronic voluntary wheel running. This aging effect aligns with data from Mumford et al. [32] who showed that gastrocnemius ORF1p protein expression

increased in an age-dependent fashion when comparing 3-month old, 12-month old, and 24-month old rats, but disagrees with the findings of Roberson et al. [43] who showed that vastus lateralis ORF1p expression did not differ between college-aged and older (58 ± 8 years old) participants. Moreover, the only study from our laboratory to show exercise reduces ORF1p expression in skeletal muscle was from Romero et al. [41] who reported that the protein trended downward with 12 weeks of resistance training in college-aged participants. Hence, again, it may be possible that resistance training, rather than endurance training in humans or voluntary wheel running in rodents, provides an adequate stimulus to downregulate ORF1p expression. While these data are insightful and open possibilities for future research comparing modes of exercise, more research is needed to determine the physiological significance of these findings.

Due to the impact of L1 activity can have on the genome, it is regulated at the DNA, RNA, and protein levels. The best characterization of regulation is through methylation of the L1 internal promoter [49]. L1 promoter methylation in the soleus and hippocampus was significantly greater in both 15-month-old SED and EX rats compared to CTL rats. This finding, along with the higher ORF1p expression levels in aged rats, is paradoxical given that we have previously shown that aging in humans and rodents is associated with a decrease in skeletal muscle L1 DNA methylation [32, 43]. However, again, this may be due to these rats being compared at 6 months versus 15 months, and this differs from the age groups compared by Mumford and colleagues as well as the human comparison by Roberson and colleagues.

An additional mechanism for L1 inhibition is through protein binding that affects L1 activity at multiple stages of the TPRT process [50]. TREX1, APOBEC3, and MOV10 are three

genes that have been shown to inhibit L1 activity, so we sought to investigate the changes in their mRNA expression in the current study. The notable changes include age-related elevations in MOV10 mRNA expression in soleus muscle and APOBEC3 mRNA expression in the liver. MOV10 interacts with the L1 RNP to degrade the L1 mRNA [34], which could play a role in the lack of a significant increase in L1 mRNA with aging observed herein. The APOBEC3 subfamily associates with ORF1p and reduces its activity [33]. Given the disparate findings between L1 DNA patterns and L1 mRNA patterns existed (i.e., no age-related elevations in L1 mRNA despite age-related elevations in L1 DNA methylation), these preliminary mRNA findings suggest that other L1 inhibitory mechanisms may have been responsible. However, these data are preliminary given that only mRNAs were assayed, and more data are needed to examine how the protein levels of these genes were affected.

The cGAS-STING pathway has been associated with increased L1 activity through an inflammatory response [16]. This cDNA-sensing pathway has various implications for disease and aging. As discussed prior, L1 mRNA can accumulate in the cytoplasm of cells in certain progeroid rodent models and this accumulation leads to heighted L1 cDNA levels. cGAS and its downstream regulator STING are activated in response to cellular cDNA accumulation, and this can lead to an inflammatory response. The only significant observation with the assayed cGAS-STING markers herein was heightened cGAS protein expression in the liver of older EX and SED rats versus CTL rats. This is difficult to reconcile because there were age-related differences in STING markers to complement these findings. However, we posit that this inflammatory pathway is more involved in cases of disease and extreme aging, and the animals

examined herein were healthy. To this effect, we have unpublished data from a human study where we compared young, healthy controls (~22 yo) to older patients (~63 yo) with end-stage osteoarthritis, undergoing either a total hip or knee arthroplasty (Osburn et al. *In review*). Notably, there was increased L1 mRNA expression in the older participants and increased ORF1p expression in the surgical leg compared to contralateral/non-surgical leg. Interestingly, STING protein levels were also upregulated in the surgical leg as well when compared to the young and contralateral biopsy samples. While these human data are preliminary because of the small sample size and limited analyses, this sets up future work to better determine if increased skeletal muscle L1 expression and an elevation in cGAS-STING markers during advanced aging and disease states contributes to localized muscle inflammation susceptibility.

Limitations

Limitations to the current study must be considered. Rodent models have proven to be useful in the context of drug discovery, longevity studies, and disease progression studies, among others, all while reducing biological variation [51, 52]. Although the rat model has been well established in research, an obvious limitation is the translatability to human physiology. The conversion to human years changes during different phases of a rat's life (i.e., a different number of rat days equates to a human year). Despite this, there is a consensus on rat age based on social maturity phases [48]. In relation to this study, 6-month-old rats are the equivalent of an 18-year-old human, while 15-month-old rats are ~40 years old in human years. Notably, female rats will reach menopause anywhere from 15-18 months of age [53]. While the EX rats performed

voluntary wheel running for a prolonged period of time in avoidance of the potential confounder of menopause, we were unable to fully investigate the aging aspects of the study given that they were only aged 15 months old. Hence, this may have been why several null findings were evident, and this requires additional interrogation. Another limitation to consider is the 9-month running intervention and large variation in the weekly running distance within EX rats. The study concluded once the average weekly running distance fell below 20 km a week for two consecutive weeks. As a result, running distances were relatively low in EX rats by this point of the study. Thus, sampling earlier in the intervention when running distances were greater may have led to more robust differences in biomarkers between groups. Finally, incomplete data for certain markers is a limitation. Of note, we were unable to assess changes in the methylation status of the L1 promoter region for the plantaris and liver, and changes in ORF1p expression in the hippocampus because of the low expression values for all these markers.

Conclusions

ORF1p protein expression increases across multiple tissues with aging in female rats, and this effect is not mitigated through 9 months of voluntary wheel running. While past studies have suggested that L1 DNA methylation changes play a role in L1 gene expression, this finding was not observed herein, and this was likely due to voluntary wheel running being an inadequate stimulus to appreciably affect L1 tissue markers. Moreover, certain cGAS-STING markers were largely unaffected by aging or voluntary wheel running, and this was likely due to animals being healthy across age groups. Future research is needed to determine how other long-term exercise

modalities in rodents (e.g., progressive treadmill training or resistance training) affects the outcomes assessed herein. Likewise, additional research is needed to examine these exercise models in older rats.

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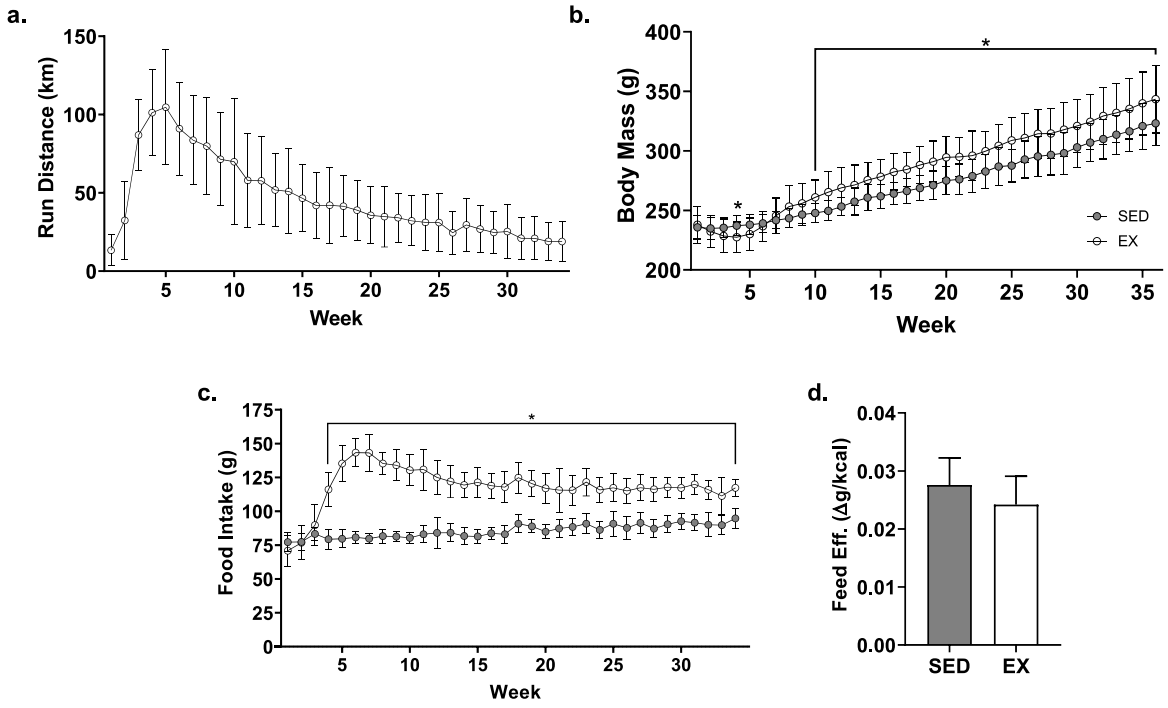
TABLES, FIGURES, AND LEGENDS

Table 1. Rat primer sequences for qPCR

Gene	Accession number
(Rat) Fibrillarin (*Fbl*; HKG) FP (5'→3') CTGCGGAATGGAGGACACTT RP (5'→3') GATGCAAACACAGCCTCTGC	NM_001025643.1
(Rat) Cyclophilin A (*Pp*ia; HKG) FP (5'→3') GCATACAGGTCCTGGCATCT RP (5'→3') AGCCACTCAGTCTTGGCAGT	NM_012583.2
(Rat) L1 (L1-3) FP (5'→3') GACCATCTGGAACCCTGGTG RP (5'→3') GGGCCTGTGTCTTGAGTTCA	DQ100473.1
(Rat) L1 (L1-Tot) FP (5'→3') GGAAGAGACCACCAACACTG RP (5'→3') GAAGGTTTAGCTCTCCCTCC	DQ100473.1 DQ100475.1 DQ100476.1 DQ100477.1 DQ100474.1 DQ100482.1
(Rat) Moloney Leukemia Virus 10 (Mov10) FP (5'→3') TCAGCAAACAGCACAGATCC RP (5'→3') AGACAAACTGGGGTGTCCAG	NM_001107711.2
(Rat) Three Prime Repair Exonuclease 1 (Trex1) FP (5'→3') CTGCCCTGAAGACCTTGGAG RP (5'→3') GGCTTCCACTGACAGATGCT	NM_001024989.1
(Rat) Apolipoprotein B MRNA Editing Enzyme Catalytic Subunit 3 (Apobec3) FP (5'→3') CTCTGTGGCGATCAGGGATC RP (5'→3') ATCTTACAGGCCCCAGGACT	NM_001033703.2

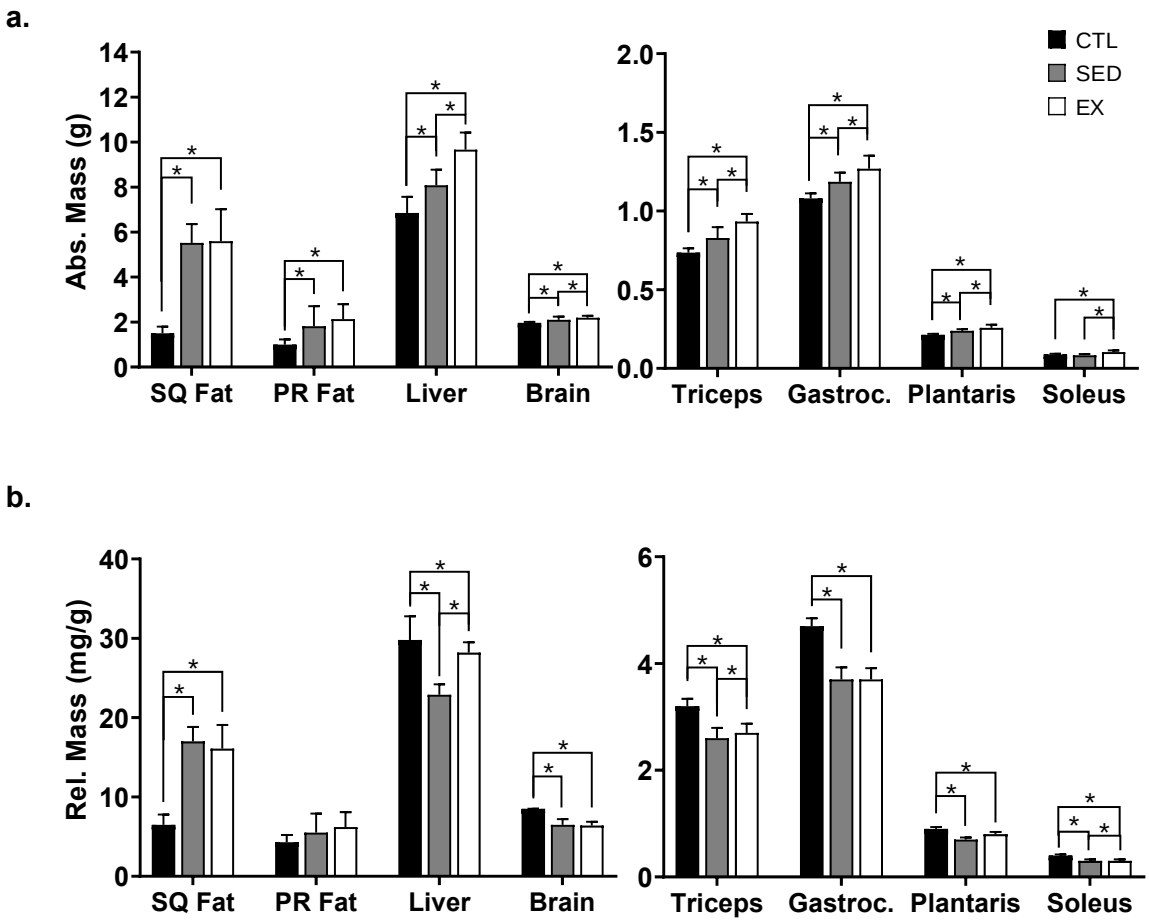
Legend: Forward and reverse primer sequences used for qPCR. Abbreviations: HKG, housekeeping gene; FP, forward primer; RP, reverse primer.

Figure 1. Weekly data collection markers



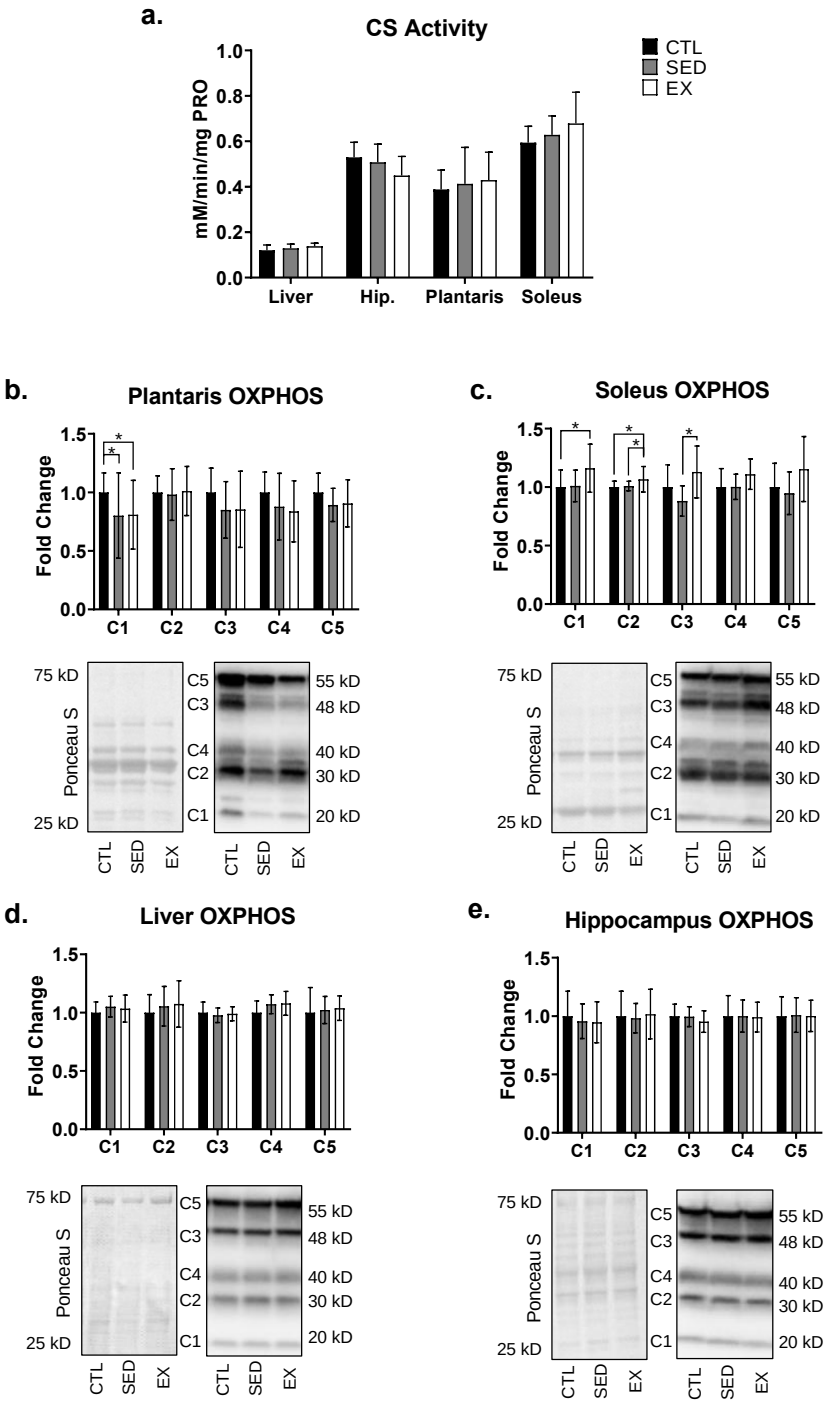
Legend: Average weekly run distance for the exercise group (panel a), average weekly body mass for sedentary and exercise groups (panel b), average weekly food intake for sedentary and exercise groups (panel c), and total average feed efficiency (panel d). Data are presented as mean ± SD, and asterisks indicate significant between-group age differences ($p<0.05$). Abbreviations: Eff, efficiency.

Figure 2. Absolute and relative tissue mass



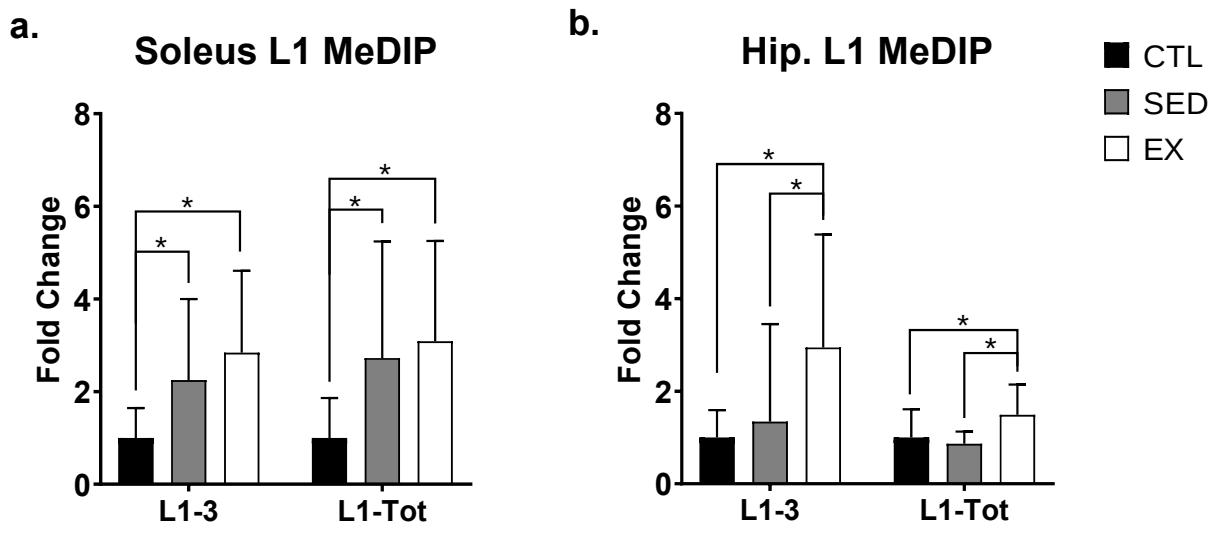
Legend: Absolute (panel a) and relative (panel b) tissue mass. Relative tissue mass was normalized to final body mass. Data are presented as mean ± SD, and asterisks indicate significant between-group age differences ($p<0.05$). Abbreviations: Abs, absolute; Rel, relative; SQ, subcutaneous; PR, perirenal; Gastroc, gastrocnemius.

Figure 3. Effects of exercise on CS activity and OXPHOS protein expression



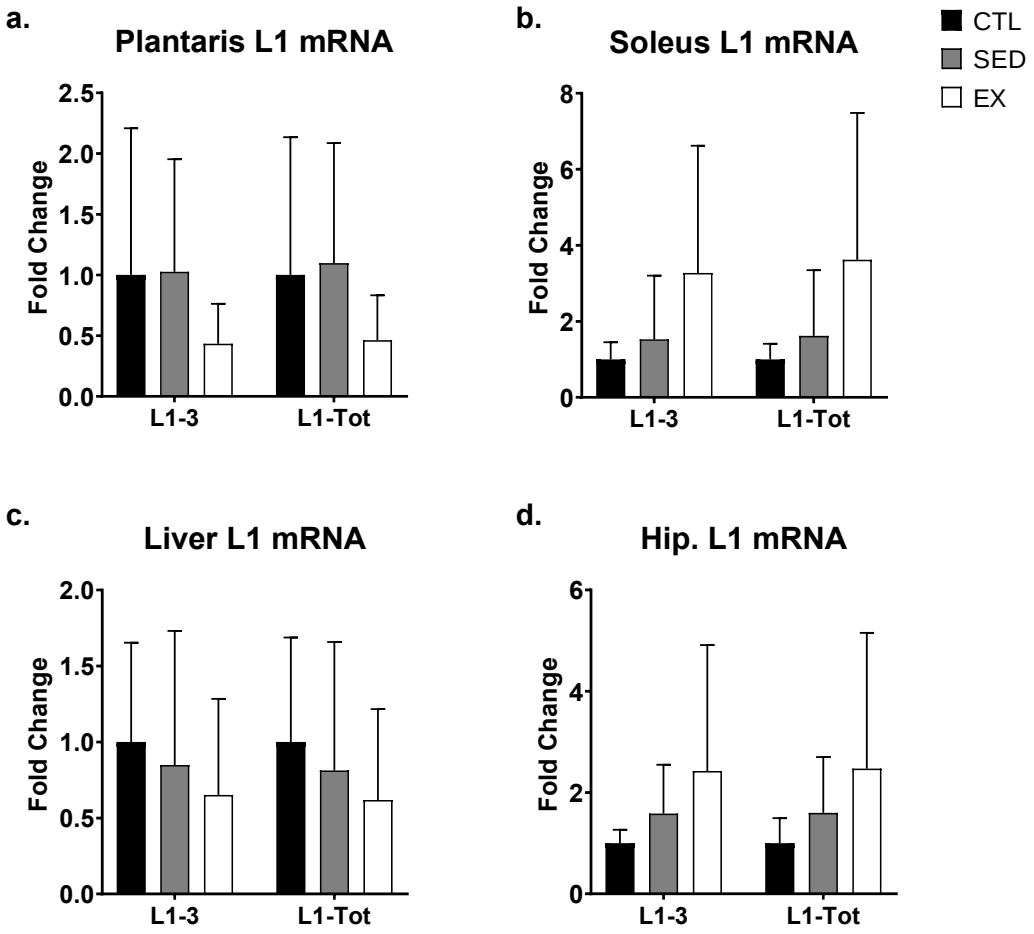
Legend: Citrate synthase activity for liver, hippocampus, plantaris, and soleus tissues (panel a). Total OXPHOS protein expression and representative images for all five complexes of the ETC in the plantaris (panel b), soleus (panel c), liver (panel d), and hippocampus (panel e). Data are presented as mean ± SD, and asterisks indicate significant between-group age differences ($p<0.05$). Abbreviations: CS, citrate synthase; Hip, hippocampus.

Figure 4. Methylation changes in the L1 promoter



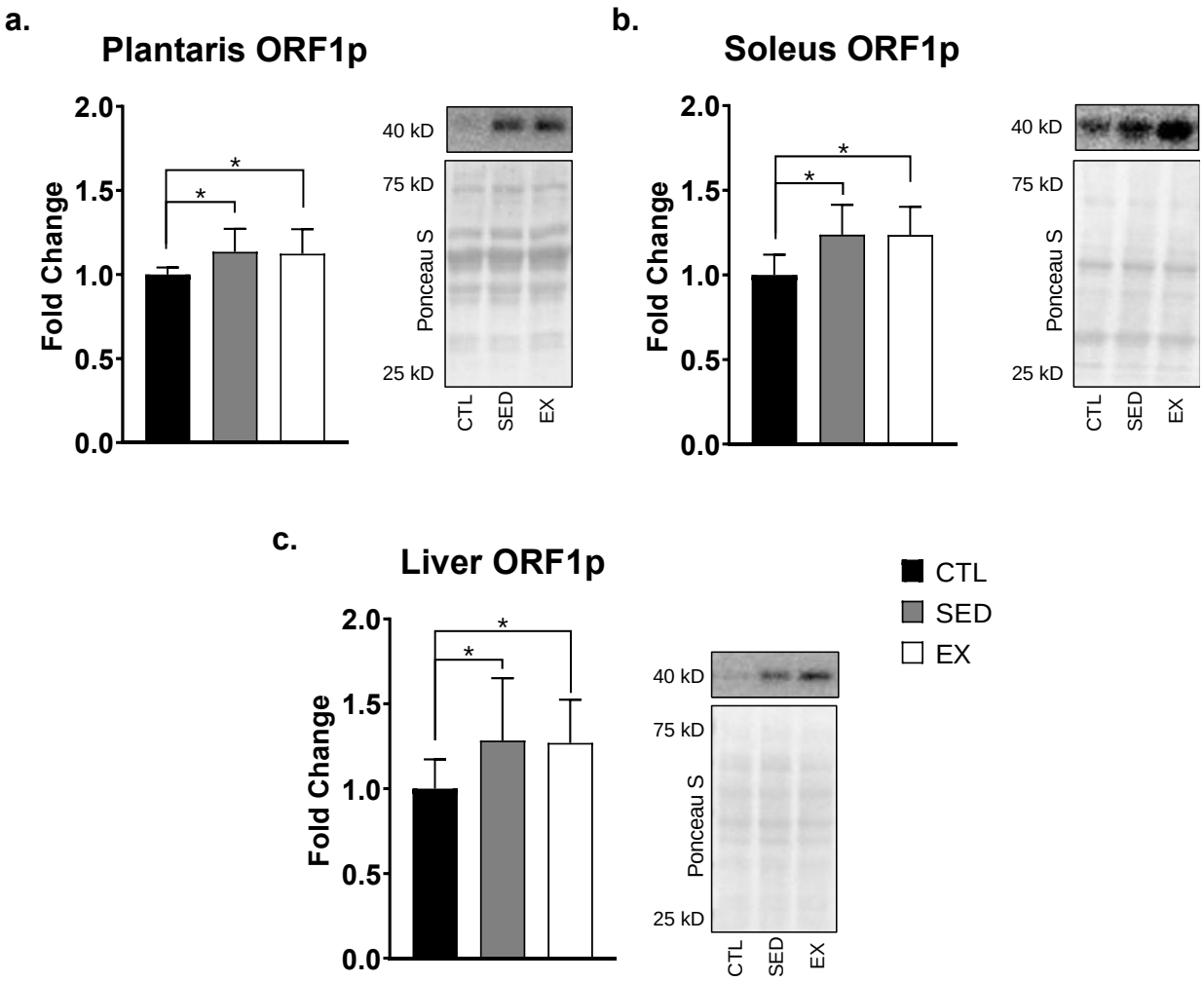
Legend: Group differences in L1.3 and L1.Tot promoter methylation for the soleus (panel a) and hippocampus (panel b). Data are presented as mean ± SD, and asterisks indicate significant between-group age differences ($p<0.05$).

Figure 5. L1 mRNA expression



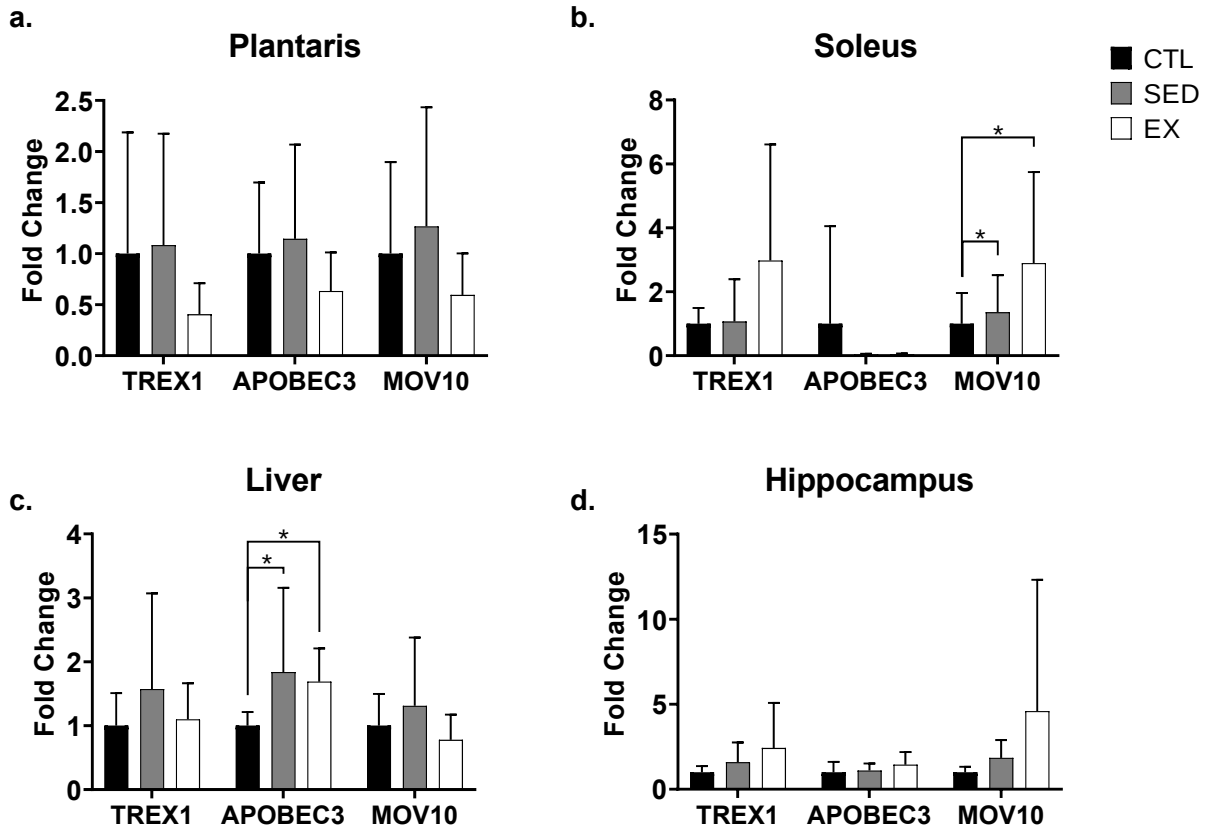
Legend: L1.3 and L1.Tot mRNA expression for plantaris (panel a), soleus (panel b), liver (panel c), and hippocampus (panel d). Data are presented as mean ± SD, and asterisks indicate significant between-group age differences ($p<0.05$). Abbreviations: Hip, hippocampus.

Figure 6. ORF1p expression



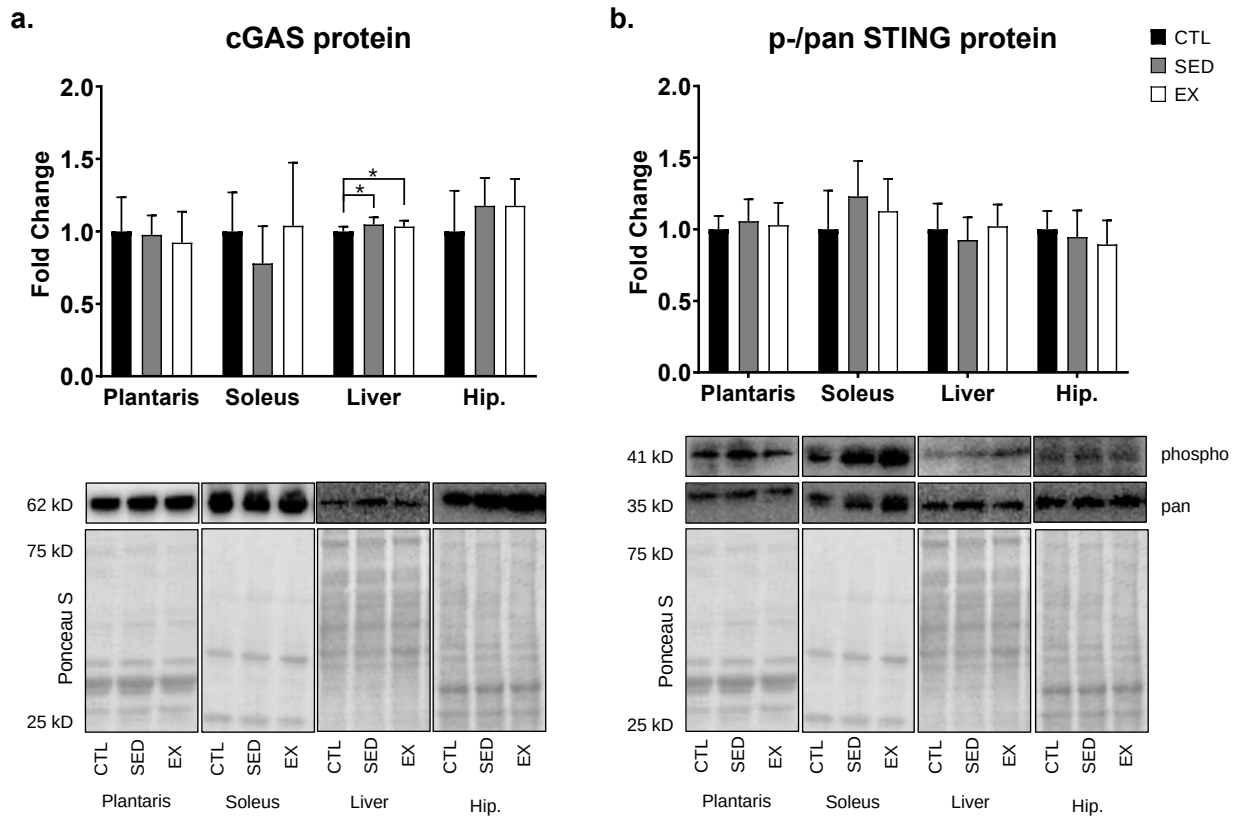
Legend: ORF1p protein expression and representative images for plantaris (panel a), soleus (panel b), and liver (panel c). Data are presented as mean ± SD, and asterisks indicate significant between-group age differences ($p<0.05$).

Figure 7. mRNA expression for regulators of L1 activity



Legend: TREX1, APOBEC3, and MOV10 mRNA expression in plantaris (panel a), soleus (panel b), liver (panel c), and hippocampus (panel d). Data are presented as mean ± SD, and asterisks indicate significant between-group age differences ($p<0.05$).

Figure 8. cGAS-STING inflammatory pathway protein expression



Legend: Protein expression and representative images for cGAS (panel a) and phospho/pan STING (panel b). Data are presented as mean ± SD, and asterisks indicate significant between-group age differences ($p<0.05$). Abbreviations: Hip, hippocampus.

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