The Role of Sarcolipin in Muscle Adaptation with Aging

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pump facilitates the transport of Ca^{2+} against a concentration gradient from the cytosol to the lumen of the SR, eliciting relaxation and maintaining Ca^{2+} homeostasis. Sarcolipin (SLN) is a small allosteric regulator of the SERCA, which when bound to the pump, lowers SERCA Ca^{2+} affinity and reduces SERCA efficiency by uncoupling the Ca^{2+} transport efforts from ATP hydrolysis. Previous research has revealed that through the regulation of SERCA activity, SLN can alter muscle function, energy expenditure, and promote Ca^{2+} signalling for metabolism, fibre type and muscle mass. While extensive research has characterized SLN expression and function in young adult male mice, few studies have examined SLN with aging and no studies have reported on SLN in female mice. Therefore, the purpose of this thesis was to examine sex and age-related differences in SLN expression and to discern the role of SLN in altering SERCA function, energy expenditure, and Ca^{2+} signalling between sexes with aging.

The first study, described in Chapter 3, explored sex-specific differences in SLN expression in young adult mice and investigated the consequences of SLN ablation on SERCA function and whole-body energy expenditure in both male and female mice. The study revealed that the soleus muscles of female mice exhibited higher levels of SLN content compared to male counterparts, which was accompanied by greater SERCA efficiency and increased adiposity with SLN ablation in females. This study reported that SLN ablation reduced whole-body energy expenditure in both male and female mice, in the absence of changes to habitual activity levels. Although females displayed relatively greater SLN content than males, the

impact of SLN ablation on absolute whole-body energy expenditure was more pronounced in males, likely attributed to their greater lean muscle mass.

The second study of this thesis (Chapter 4) had two main aims: first, to investigate sex-related variations in SLN expression with aging, and second, to examine if SLN may offer SERCA protection against age-relative oxidative damage and dysfunction. With respect to the first aim, this study found that SLN expression increased with age and that the sex differences in SLN expression persisted with age, with females displaying greater SLN expression in soleus muscles across age groups. In order to examine the role of SLN in the protection of SERCA with aging, measures of SERCA function and SERCA damage were assessed in the soleus muscles of wild type (WT) and SLN ablated ($Sln^{-/-}$), male and female mice at two ages, 4-6 months and 18+ months, reflecting young adult and older adults, respectively. Contrary to the hypothesis, SLN ablation had no impact on SERCA nitrosylation with aging and SLN ablation appeared to inhibit SERCA function in a manner similar to young adults. Additionally, the research uncovered sex-specific differences in nitrosylation damage to SERCA1a, with females displaying greater damage to SERCA1a than males. This data challenges the prevailing notion that SERCA2a is more susceptible to age-related oxidative damage, as previous studies have exclusively examined males.

The final study (Chapter 5) of this thesis examined whether the upregulation of SLN with aging activated calcineurin (Cn) signalling and promoted greater muscle mass and muscle function. Similar to findings in Chapters 3 and 4, SLN expression was higher in the soleus muscles of both females and older animals compared to male and young adult counterparts.

This study aligned with several established sex and age-differences in the literature. Mainly, females and older animals displayed lower muscle mass, absolute force and fewer type II fibres than males and young adult, respectively. In contrast to the hypothesis, SLN ablation had no discernible impact on Cn signaling, muscle mass, or maximal muscle function, suggesting the upregulation of SLN with aging may not serve the same role in promoting beneficial Ca^{2+} signaling as observed previously in various unloading and disease models. While SLN ablation did not impact Cn signalling, this study revealed novel evidence for the activation of Cn signalling in skeletal muscle with age. This may align with previous research finding elevations to resting cytosolic [Ca^{2+}] in aged muscle and future research should directly target Cn in order to discern the impact on muscle mass, fibre type and function with aging between sexes.

The collective results of the studies presented here confirm that SLN expression increases with age in both male and female mice and aids in clarifying the role of SLN in aging muscle. Furthermore, the studies of this thesis sought to begin addressing the sex bias within SLN research and revealed the consistent and novel evidence that SLN expression is greater in females. The sexual dimorphism in SLN expression make the argument for the use of female mice in studies examining SLN and provide foundational evidence for future research examining the impact of SLN on muscle metabolism between sexes.

Acknowledgements

First and foremost, I'd like to thank my family for their love and support. To my mom and dad, every accomplishment I've achieved is for you and because of you. Thank you for reassuring me, roasting me, and loving me through it all.

I also need to thank my supervisor, Dr. Russell Tupling. I'm not sure where to start in thanking you for the experience of my graduate studies. I feel very lucky to have done both my MSc and my PhD with a supervisor who so honestly cares about his students and who has such a passion for research and learning. I'm more grateful than I think you know.

Similarly, I must thank my lab mates, past and present: Val, Dan, Cat, Emma, Mark, Adi – I truly valued our time together. I enjoyed learning from and with you in the lab and laughing and cracking a beer with you outside the lab. Additionally, the students throughout the physiology labs, you helped ease the burden of the hard days and I appreciate you. While I know the nature of graduate studies and academia can spread people out to difference universities, I hope our paths cross again ☺.

To my partner, Derek, thank you for supporting me across this finish line. I love you and I can't wait to see what the next chapter of our life looks like. And finally, to my best friend Dr. Kayleigh Beaudry. I could not have done this without you. The times when science didn't work, rejections were received, samples were thrown out, and a global pandemic hit were made so much brighter because you were there with me to laugh-cry through it.

To you all,

Thank you!

Dedication

This doctoral thesis is dedicated to my grandfather, Tony Miller. As my grandfather and an elementary school principal, you were the first educator in my life. It's you I must thank for sparking a sense of curiosity in me that led to me ask why/how so many times that I ended up in graduate school. I love and miss you deeply.

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List of Abbreviations

$[Ca^{2+}]_{cyt}$ – cytosolic calcium concentration

AMPK – AMP-activated protein kinase

BAT – brown adipose tissue

Ca^{2+} - calcium

CAMK – Ca^{2+}-calmodulin–dependent protein kinase

Cn – calcineurin

CNM – centronuclear myopathy

CSQ – calsequestrin

DHPR – dihydropyridine receptor

DMD – Duchenne muscular dystrophy

EDL – extensor digitorum longus

***Mdx* –** murine model for Duchenne muscular dystrophy

MEF2 – myocyte enhancing factor 2

MLN – myoregulin

NFAT – nuclear factor of activated T-cells

NST – non-shivering thermogenesis

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

PLN – phospholamban

RYR1 – ryanodine receptor

SERCA – sarco(endo)plasmic reticulum Ca^{2+} ATPase

SLN – sarcolipin

***Sln*$^{-/-}$ –** sarcolipin knockout

***Sln*^{OE}** – sarcolipin overexpressing

WT – wild type

Chapter 1: Introduction

1.1 The Regulation of Calcium by SERCA within Skeletal Muscle

Calcium plays a complex yet essential role in skeletal muscle as both an initiator of muscle contraction and as a signalling molecule for muscle adaptation. During rest, the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) remains low (≤100nM) and is tightly regulated by the sarcoplasmic reticulum (SR) and several Ca^{2+} handling proteins (1). The SR, a tubular organelle, which surrounds each myofibril, acts as the major storage site for Ca^{2+} within skeletal muscle. In contrast to the cytosol, SR luminal $[Ca^{2+}]$ can reach up to 11mM and 21mM in slow and fast twitch fibres, respectively, with the help of the high capacity, low affinity Ca^{2+} binding protein, calsequestrin (CSQ) (2, 3). When an action potential occurs, dihydropyridine receptors (DHPR) along the T-tubule of the sarcolemma act as voltage sensors triggering the opening of the SR Ca^{2+} channels. When these SR Ca^{2+} channels, or ryanodine receptors (RYR), open, Ca^{2+} disassociates from CSQ rapidly flooding the cytosol where it binds and relieves the inhibition on contractile myofibrils, ultimately resulting in force production. When an action potential is terminated and RYR channels close, Ca^{2+} is then pumped back into the SR via sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) pumps, resulting in muscle relaxation. In addition to being the primary agent in muscle relaxation, SERCA pumps also play an important role in regulating cellular Ca^{2+} concentrations, both to ensure an adequate SR Ca^{2+} load for subsequent contractions and maintaining low $[Ca^{2+}]_{cyt}$ in the face of SR Ca^{2+} leak. Consequently, any alterations to SERCA function resulting in changes to $[Ca^{2+}]_{cyt}$ would directly impact muscle performance and may alter downstream Ca^{2+} signalling for muscle adaptation.

1.1.1. The Role, Structure and Function of SERCA Pumps

SERCA pumps are highly conserved 110kDa P-type ATPases embedded in the longitudinal SR membrane that are responsible for SR Ca^{2+} uptake and muscle relaxation. The SERCA family is encoded by *ATP2A1-3* genes which respectively code for the expression of SERCA1, SERCA2 and SERCA3 isoforms, with further isoform diversity occurring through alternative splicing (4). In mature skeletal muscle specifically, the two main SERCA isoforms of interest are SERCA2a expressed in slow twitch type I fibres and SERCA1a expressed predominantly in fast twitch type II fibres (5). Although there is little difference in the kinetics between these SERCA isoforms (6), differences in SERCA content can result in significant differences in SR Ca^{2+} uptake and relaxation rates. Specifically, this can be witnessed between muscle fibre types where fast twitch fibres display ~5 fold greater SERCA content accompanied by faster rates of Ca^{2+} uptake and muscle relaxation compared to slow twitch fibres (7).

Structurally, SERCA is comprised of 10 transmembrane α-helices (M1-M10) connected to a large cytosolic headpiece containing three cytoplasmic domains (actuator, nucleotide binding and phosphorylation domains) (8). SERCA has two Ca^{2+} binding sites located within a transmembrane groove created by α-helices M4, M5, M6 and M8 (9). In order to transfer Ca^{2+} from the cytosol to the SR, SERCA undergoes a cycle of conformational changes driven by ATP hydrolysis (see Figure 1). The catalytic cycle begins with SERCA in an E1 state in which the two high affinity Ca^{2+} binding sites are exposed to the cytosol. Upon the binding of Ca^{2+} and ATP, the ATP-bound E1-2Ca^{2+} complex undergoes conformational change bringing the nucleotide binding domain adjacent to the phosphorylation domain (10, 11). The subsequent hydrolysis of ATP induces the formation of a phosphorylated SERCA

intermediate, E1~P-ADP-2Ca^{2+} which drives a structural transition to phosphorylated E2P-2Ca^{2+} where the opening to the transmembrane domain inverts towards the SR lumen (10). This transition also results in lower SERCA affinity for Ca^{2+} allowing for the disassociation of Ca^{2+} into the SR lumen. After Ca^{2+} disassociation, E2P is recycled back to the E1 state by the dephosphorylation of SERCA (12-14). Given this process, the optimal coupling ratio for SERCA is the transfer of two Ca^{2+} ions to one molecule of ATP, albeit SERCA activity and the stoichiometry of Ca^{2+} transport is regulated by post-translational modifications and by the presence of specific allosteric protein modulators.

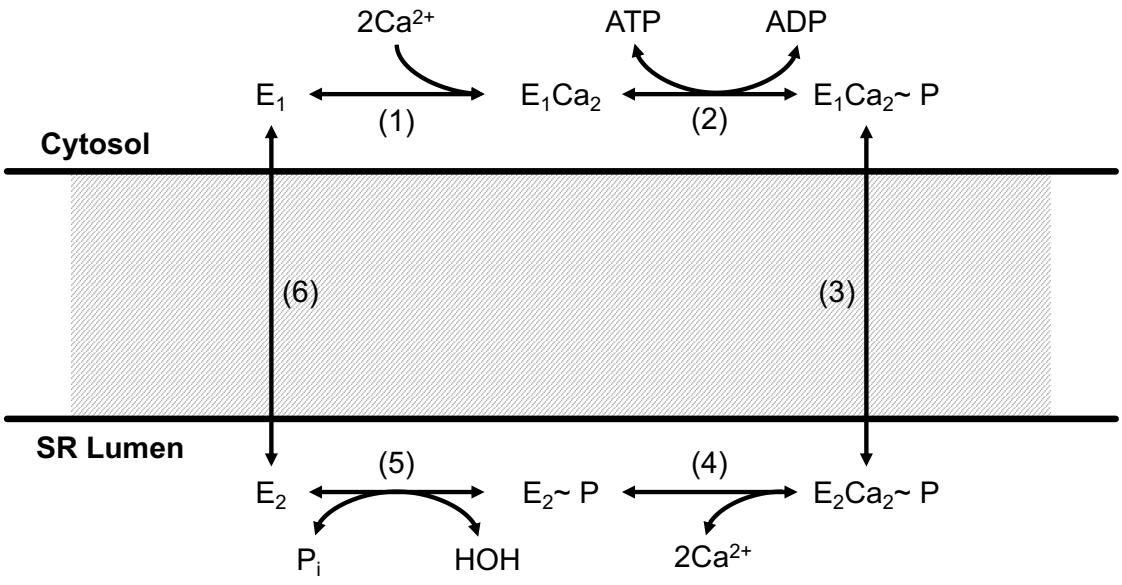


Figure 1. Schematic of the Catalytic Cycle of SERCA.

(Figure redrawn from Inesi, 1985; de Meis and Vianna, 1979). 1. From its original state (E_1), two Ca^{2+} ions bind to SERCA (E_1Ca_2) 2. Upon ATP binding, SERCA undergoes conformational change resulting in ATP hydrolysis (E_1Ca_2 ~P) 3. This phosphorylation on SERCA drives in further conformational change resulting in an inversion which exposes the Ca^{2+} binding sites to the lumen of the SR (E_2Ca_2 ~P) 4. Here, reductions in Ca^{2+} affinity result in the dissociation of Ca^{2+} into the SR lumen (E_2 ~P). 5-6 Dephosphorylation then occurs which drives SERCA back to its original conformation (E_1).

1.1.2 SERCA Regulatory Proteins

While SERCA activity is primarily regulated by fluctuations in $[Ca^{2+}]_{cyt}$, there are now several known regulatory partners. The majority of these SERCA regulatory proteins work to inhibit SERCA activity and SR Ca^{2+} uptake, including sarcolipin (SLN) and phospholamban (PLN), and the more recently discovered myoregulin (MLN), endoregulin (ELN) and another-regulin (ALN) (16, 17) (see Figure 2). Structurally, these SERCA inhibitory proteins are small single span proteins ranging from 31 to 65 amino acids, which all share key amino acids within their transmembrane region yet vary considerably in both the length and amino acid sequencing of their N-terminal cytosolic regions. When examining skeletal muscle specifically, PLN, SLN and MLN are the primary SERCA inhibitory proteins of interest, as ALN and ELN have been shown to inhibit SERCA2b and SERCA3 isoforms which are expressed predominantly in smooth muscle and in non-excitable cells, respectively (16). Functionally, each of these single span micropeptides have been shown to inhibit SERCA activity by binding directly to the transmembrane region of SERCA (18, 19). When bound to SERCA, the inhibitory proteins, SLN, PLN and MLN, reduce SERCA's apparent affinity for Ca^{2+}, effectively slowing the rate of Ca^{2+} uptake into the SR (18, 20, 21). Correspondingly, studies in which SLN, PLN or MLN have been ablated from otherwise healthy skeletal muscle, show improvements in SR Ca^{2+} uptake accompanied with faster relaxation rates (18, 22-24).

There is also considerable variation in the expression pattern of the SERCA inhibitory proteins across muscle groups and fibre types, generally aligning with preferential inhibition of specific SERCA isoforms (16). For instance, human and animal studies have consistently found that PLN preferentially binds and follows the expression pattern of SERCA2a, with

expression highest in cardiac and slow twitch muscles (25-28). MLN, originally discovered in non-coding RNA by Anderson et al (2015), preferentially binds and inhibits SERCA1 (18). While mRNA expression of MLN was originally found in all skeletal muscle, protein content and expression across individual fibre types has yet to be reported (18). The expression pattern of SLN is slightly more complicated. While SLN has the ability to modulate the activity of both SERCA1a and SERCA2a in HEK-293 cells (29), its expression *in vivo* suggests the primary role of SLN is in modulating SERCA1a specifically within type IIA fibres. Indeed, single fiber Western blot analyses on human vastus lateralis biopsies showed clearly that SLN is co-expressed with SERCA1a primarily in fast-twitch type IIA muscle fibers, although a small percentage of slow-twitch type I fibers also co-express SLN and SERCA1a (28). While larger animals, such as rabbits and dogs, show SLN expression in fast twitch muscles similar to humans (30), SLN expression in mice is confined to slower oxidative muscles (27), albeit this is likely due to variations in muscle fibre type composition across species. In mice, SLN is expressed in slow twitch muscles, such as soleus, red gastrocnemius and diaphragm, comprised predominantly of type I and type IIa fibers, and absent from glycolytic fast twitch muscle, comprised primarily of type IIX and IIB (22, 27, 31). In fact, type IIA fibers account for 40-50% of the total fibers within mouse soleus and red gastrocnemius, whereas type I fibers only account for 10-30% of the fibers in these muscles (32, 33). Further, in the mouse soleus specifically the ratio of SERCA1a:SERCA2a is ~14:1 with over 90% of the SERCA molecules being SERCA1a (34), collectively suggesting again that, while the expression across muscle groups may vary between species, SLN primarily regulates SERCA1a in type IIA fibers. However, it should be noted that SLN has been shown to increase the binding capacity of PLN to SERCA2a when co-expressed in HEK-293 cells (29), causing additive SERCA inhibition.

While it is unclear as of yet whether this occurs *in vivo*, it may be physiologically relevant in type I fibers or in atria where SERCA2a, SLN and PLN are all co-expressed and the deletion of SLN improves SERCA function (28).

Just as genetic ablation of PLN, SLN and MLN improves SERCA function, dissociation of these inhibitory proteins from SERCA also improves SERCA function. For example, at elevated $[Ca^{2+}]_{cyt}$, PLN has been shown to have a lower affinity and diminished cross linking capacity for SERCA allowing for full activation of the pump (35). Potentially compounding with lower affinity, phosphorylation of PLN by Ca^{2+}-activated kinases also results in dissociation from SERCA. Unlike SLN, PLN has an extended N-terminal cytoplasmic region which has been shown to be important both to its SERCA inhibitory function and phosphorylation status. In fact, PLN has been shown to be phosphorylated at three distinct locations, Ser10 by protein kinase C (PKC), Ser16 by cAMP-dependent protein kinase (PKA) and at Thr17 by Ca^{2+}-calmodulin–dependent protein kinase (CAMK) (36, 37). When monomeric PLN is phosphorylated, it dissociates from SERCA, relieving its inhibition, and forming inactive PLN pentamers. This process has been extensively researched within cardiac tissue where decreased PLN phosphorylation and an increased PLN/SERCA2a ratio are hallmarks of cardiomyopathy and heart failure (38). Although phosphorylation of MLN and any resulting changes to SERCA activity have yet to be reported on, the similar N-terminal structure of MLN and PLN may suggest that MLN can be similarly regulated. With respect to SLN, phosphorylation and the resulting impact on SERCA function are less clear. Although SLN lacks an extensive N-terminal region, Bhupathy et al (2009) found that CAMKII phosphorylated SLN at Thr5 in rat cardiomyocytes *in vitro* (39). However, this work is refuted

by others who have found no evidence of SLN phosphorylation when co-expressed with SERCA1 in HEK-293 T-cells or in native rabbit SR vesicles (20). Given the reduced N-terminal region and the discrepancies in the literature, it is unlikely phosphorylation regulates the interaction between SLN and SERCA to the same extent as PLN phosphorylation.

Recent work has also uncovered allosteric regulatory proteins which promote SERCA function. Dwarf open reading frame (DWORF), a 34 amino acid peptide discovered by Nelson et al (2016), enhances SERCA activity and Ca^{2+} uptake, not only by displacing SLN, PLN, and MLN from the pump (40), but also by directly activating SERCA in the absence of other regulators (41). While *in vitro* work has shown DWORF is capable of binding SERCA1, 2 and 3 isoforms, *in vivo* DWORF protein expression is exclusive to cardiac and slow twitch muscle in rodents and has yet to be discerned in larger mammals and humans (40). Small ankryin 1 (sAnk1), a protein that shares transmembrane amino acid sequence similarities to SLN, can also modulate SERCA activity. Although sAnk1 alone reduces SERCA activity, when co-transfected with both SERCA1 and SLN, sAnk1 improves SERCA activity by binding and blocking SLN inhibitory function (42, 43). As many of these SERCA regulatory proteins have only recently been discovered, further research on the interplay between them will be required in the coming years to develop a clearer and more refined understanding of SERCA regulation.

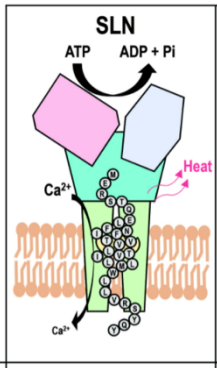
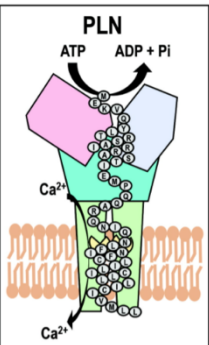
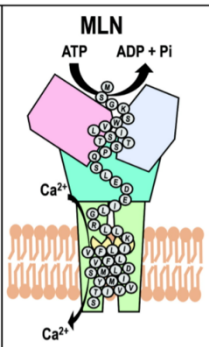
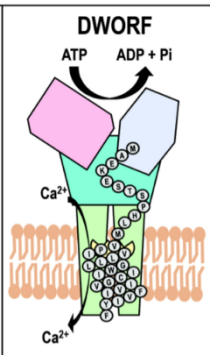
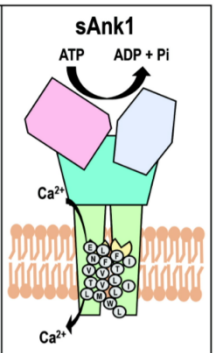
	SLN	PLN	MLN	DWORF	sAnk1
					
SERCA V_{max}	No Change	No Change	No Change	No Change	No Change
SERCA K_{Ca}	Increased	Increased	Increased	Recovered*	Recovered†
Coupling Ratio	Decreased	No Change	Unknown	Unknown	Unknown
Tissue Distribution	Cardiac (Atria) and Oxidative (mouse) or Glycolytic (Human) Skeletal Muscle	Cardiac (Left Ventricle) and Oxidative Skeletal Muscle	All Skeletal Muscle (Predominantly Glycolytic Skeletal Muscle)	Cardiac and Oxidative Skeletal Muscle	Oxidative Skeletal Muscle
SERCA Isoform Association	**SERCA1a**/SERCA2a	SERCA1a/**SERCA2a**	**SERCA1a**/SERCA2a	SERCA1a/**SERCA2a**	SERCA1a

Figure 2. Graphical Summary of SERCA Regulatory Proteins

Originally created and published by Gamu et al (2020). Schematic figures depict amino acid sequences of the SERCA regulatory proteins, SLN, PLN, MLN DWORF and sAnk1, and their interaction with SERCA. Changes to maximal activity (V_{max}), Ca^{2+} affinity (K_{ca}), and efficiency (Ca^{2+} - ATP coupling ratio) of SERCA in the presence of the regulatory protein is listed below each corresponding schematic, along with tissue distribution and preferential SERCA isoform association. * DWORF recovers SERCA Ca^{2+} affinity by displacing SLN, PLN and MLN from SERCA, but DWORF alone does not alter SERCA activity. † Similarly, by displacing SLN from SERCA, sAnk1 recovers SERCA Ca^{2+} affinity.

1.2 Sarcolipin in Muscle Performance, and Adaptation

Sarcolipin plays a unique role in modulating muscle function, as it is the only SERCA regulatory protein known to alter SERCA efficiency in addition to SERCA Ca^{2+} affinity. When bound to SERCA, SLN causes Ca^{2+} disassociation or "slippage" from the pump during its catalytic cycle resulting in delayed Ca^{2+} uptake as well as higher energy expenditure and heat production (44-46). In contrast, recent evidence has shown that the N-terminal domain of PLN blunts its ability to uncouple SERCA function (47) and, while the uncoupling capabilities of MLN are yet to be reported, the extended N-terminal region of MLN may suggest that it too is incapable of altering SERCA efficiency. Reductions in the affinity and efficiency of SERCA-SLN alter cytosolic Ca^{2+} which impacts muscle function directly by slowing relaxation and promoting sustained cross bridge formation. Indeed, soleus muscle from transgenic SLN knockout ($Sln^{-/-}$) mice display the expected increase in SERCA function, SR Ca^{2+} uptake and relaxation rates, however the ablation of SLN also reduces twitch force production (22, 48). Accordingly, the transgenic overexpression of SLN (Sln^{OE}) in mouse extensor digitorum longus (EDL), where SLN is often undetectable, results in increased twitch force production (49). These changes are presumably due to the direct impact of SLN slowing SERCA function and amplifying twitch Ca^{2+} transients, as the gains in force generation with SLN expression are lost at higher frequencies with saturating Ca^{2+} transients. More recently, several groups have also begun to assess how SLN may affect muscle function indirectly by activating downstream Ca^{2+} and AMP related signaling for muscle remodeling. The remainder of this section will review the role for SLN in promoting the adaptation of muscle metabolism, fibre type and mass.

1.2.1. Sarcolipin, SERCA Inefficiency and Metabolism

The process of muscle relaxation and SR luminal Ca^{2+} pumping by SERCA is ATP dependent and thus metabolic in nature. Several studies have sought to determine the contribution of SERCA activity to resting skeletal muscle metabolic rate, with estimates varying from 18 - 48% (34, 50, 51). In a study conducted by Smith et al (2013) using indirect calorimetry, SERCA activity comprised 42-48% of resting muscle metabolic rate, which when extrapolated to the whole body was calculated to account for 15-20% of total daily energy expenditure (34). Given the unique ability of SLN to uncouple SERCA Ca^{2+} transport from ATP hydrolysis, it has been extensively reviewed as an agent for increasing metabolic expenditure and thermogenesis (17, 52-55). Ablation of SLN in mice has been shown to lower the relative contribution of SERCA activity to resting energy expenditure by 13% compared to WT mice (56), albeit this finding was not accompanied by any significant changes to body weight or daily whole body VO_2 between *Sln*$^{-/-}$ and WT animals (48). Although these findings may suggest that SLN does not significantly impact whole body energy expenditure, there is evidence to suggest that there is a greater role for SLN when faced with metabolic challenges, including exercise. Indeed, while whole body VO_2 did not differ between WT and *Sln*$^{-/-}$ mice over a 24-hour aggregate, when examining only the 12-hour dark phase in which mice are active VO_2 was lower in *Sln*$^{-/-}$ mice despite similar ambulatory activity (57). Furthermore, while there were no differences in VO_2 max, *Sln*$^{-/-}$ mice have been shown to expend less energy throughout submaximal treadmill exercise than WT counterparts, suggesting that at least at submaximal activity levels the presence of SLN promotes energy expenditure (57).

Research examining other metabolic challenges, such as caloric and climate stress, have helped solidify the role for SLN in metabolism. In fact, several groups have found SLN to be protective against diet-induced obesity. Bal et al (2012) found that $Sln^{-/-}$ animals displayed greater adiposity via MRI and fat pad weight post 12-week high fat diet despite consuming less food than high fat fed WT counterparts. Similarly, in a study by Gamu et al (2015), an 8-week high fat diet resulted in increased adiposity and glucose intolerance in $Sln^{-/-}$ animals greater than that of WT counterparts (54). In accordance with SLN being protective against diet induced obesity, SLN protein content has been shown to be adaptive in response to caloric stress. Bombardier et al (2013) found an 8-week high fat diet resulted in greater weight gain and adiposity in $Sln^{-/-}$ than WT animals and that SLN protein expression increased 3-fold in high fat fed WT animals compared to chow fed WT controls (57). The dynamic increase in SLN content with high fat feeding is thought to occur in order to promote futile SERCA activity and increase energy expenditure. Moreover, primary myocytes derived from human muscle biopsies show greater SLN mRNA and protein expression in obese individuals compared to lean suggesting the findings in mice may also translate to humans (58).

Finally, through its ability to uncouple SERCA and promote ATP hydrolysis, SLN has been examined as an agent for protection against cold stress. In response to cold exposure, mammals undergo adaptive thermogenesis, increasing metabolic energy expenditure through shivering and non-shivering thermogenesis (NST) (59). This mechanism is especially important for infants and small animals, whose body surface to volume ratio is greater allowing for faster heat dissipation. Traditionally, NST was thought to occur exclusively through uncoupling protein 1 (UCP1) in brown adipose tissue (BAT) (60). UCP-1 is a mitochondrial

uncoupling protein which increases heat production by increasing proton leak and altering the proton gradient in the mitochondrial intermembrane space. In response, greater substrate oxidation, and thus heat production, is required to restore the electrochemical gradient (61). More recently, research groups have sought to examine NST mechanisms outside UCP-1 in BAT as humans and larger mammals have limited quantities post infancy and emerging evidence suggests SLN could promote NST (17, 62). In accordance with its ability to uncouple SERCA Ca^{2+} pumping from ATP hydrolysis, greater heat production is witnessed when the ratio of SLN to SERCA is increased (46). In a study by Bal et al (2012), the authors sought to examine the role of SLN in NST by surgically removing BAT from WT and $Sln^{-/-}$ mice, effectively removing the contribution of BAT UCP-1 to NST (52). Interestingly, when exposed to cold (4°C) the BAT ablated $Sln^{-/-}$ mice displayed a reduction in core temperature and hypothermia with nearly 85% of animals reaching the ethical criteria for early protocol removal (core temperature of 25°C) (52). This advanced sensitivity to cold was not found in BAT ablated WT counterparts who were able to maintain core temperatures, clearly displaying that SLN, through its promotion of inefficient SERCA function, plays a role in NST (52). While it should be noted that $Sln^{-/-}$ mice with intact BAT were able to maintain core body temperatures similar to WT (52), the findings in BAT ablated mice may lend insight into larger mammals with naturally limited BAT stores. Together the data from exercise, diet and cold exposure demonstrate the role for SLN in metabolism and that dynamic regulation of SLN content is harnessed in order to maintain metabolic homeostasis during times of cell stress.

1.2.2. Sarcolipin and Calcium Signalling in Metabolism

While it is clear that SLN plays a role in promoting energy expenditure during times of cell stress, multiple research groups have also sought to examine if by altering $[Ca^{2+}]_{cyt}$ and energy homeostasis SLN may activate cell signalling for downstream metabolic remodeling. While studies assessing adult *Sln*$^{-/-}$ mouse soleus have consistently shown SLN ablation has no impact on mitochondrial DNA content or on the expression levels of oxidative phosphorylation proteins (63, 64), there is dissenting evidence on mitochondrial function. Gamu et al (2017) found that SLN ablation had no effect on the maximal activity of mitochondrial enzymes succinate dehydrogenase (SDH), cytochrome-c oxidase (COX), citrate synthase (65) or β-hydroxyacyl-CoA dehydrogenase (β-HAD) within soleus muscle (63). Conversely, while Maurya et al (2018) also found no change in mitochondrial content with SLN ablation (56), *Sln*$^{-/-}$ soleus expressed lower levels of enzymes involved in lipid oxidation (lipoprotein lipase protein (LPL), carnitine palmitoyl transferase 1 (CPT1), long chain acyl-CoA dehydrogenase (LCAD), and trifunctional enzyme subunit beta (HADHB)), accompanied by reduced fatty acid oxidation, suggesting SLN may alter substrate utilization (64). While these modest phenotype adaptations may suggest that the ablation of SLN, and the accompanied improvements in SERCA activity, do not result in metabolic adaptation, it may also be indicative of the relatively low endogenous levels of SLN expression in mouse soleus.

As such, studies examining models with higher SLN expression have been useful to discern its role in cell signaling (56, 64, 66). Specifically, transgenic *Sln*^{OE} results in significant improvements in treadmill exercise endurance, along with enhanced fatigue resistance in isolated soleus and EDL muscles *in vitro*, compared to WT controls (49). This improvement

in fatigue resistance is thought to be due to elevated fatty acid metabolism as SLN^{OE} muscles show increases in expression of fatty acid transport proteins, enzymes involved in lipid oxidation and higher rates of fatty acid oxidation compared to WT animals (49, 64). Moreover, in a study by Maurya et al (2018), upregulation of SLN in myotubes triggered mitochondrial biogenesis by raising $[Ca^{2+}]_{cyt}$ levels, which activated CAMKII and PGC-1α (64). Altogether, these studies suggest that overexpression of SLN triggers adaptive metabolic remodeling by activating CAMKII to improve muscle energetics and enhance muscle contractility and fatigue resistance. In contrast to these positive effects of chronic overexpression of SLN, abrupt overexpression of SLN induced via electroporation of SLN cDNA, results in decreased SERCA activity, relaxation rate, peak force generation, as well as increased fatigability in rat soleus (67). While reductions in SERCA function are expected with an increase in SLN, the discrepancies in muscle performance may be explained in part by Ca^{2+} signaling as a singular abrupt increase in SLN by electroporation may not be sufficient to induce the downstream Ca^{2+} signaling and mitochondrial adaptations witnessed with chronic transgenic SLN overexpression.

Examining developing muscle also revealed findings similar to that of the chronic SLN overexpression in *Sln^{OE}* mice. It is well known that SLN is naturally upregulated severalfold during muscle development in both fast and slow twitch muscles (27). This upregulation is witnessed in neonatal muscle and peaks around birth, after which SLN remains present only in oxidative mouse muscles (68). The purpose of this upregulation in SLN during development seems multifaceted. Due to the thermogenic nature of the interaction between SLN and SERCA, the upregulation of SLN during development is thought to be a protective mechanism

promoting the adaptive thermogenesis discussed previously. In line with this theory, cold adaptation in neonates prevents the downregulation of SLN witnessed with maturation suggesting the developmental upregulation of SLN is at least in part thermogenic in nature (68). However, developmental upregulation of SLN also seems to play a role in the adaptive metabolic remodeling mentioned above, as SLN ablation from developing quadriceps results in lowered fatty acid oxidation (64). Additionally, while studies have found no change to mitochondrial content in adult $Sln^{-/-}$ mice, muscles from developing $Sln^{-/-}$ mice display reduced mitochondrial DNA and abnormal mitochondrial cisternae, further suggesting that SLN plays a role in mitochondrial function and metabolic profile of muscle (64). This phenomenon is also apparent in differentiating myotubes which naturally and temporally upregulate SLN. Genetic deletion of SLN in differentiating myotubes resulted in lower mtDNA, lower rates of fatty acid oxidation and reduced oxygen consumption rates (64). It should be noted that while SLN deletion resulted in reduced mitochondrial content and function in developing muscle, AMPK phosphorylation was increased in developing $Sln^{-/-}$ muscle (64). This is counterintuitive to the role of SLN in promoting SERCA ATP consumption and suggests it is unlikely that SLN works to mediate metabolic adaptation by activating AMPK. Together the evidence provided from developing muscle and Sln^{OE} mice, suggests that by slowing SR Ca^{2+} uptake and activating CAMKII and PGC-1α, SLN can enhance oxidative metabolic capacity in muscle. With this in mind, the downregulation of SLN in adult fast twitch glycolytic muscles seems physiologically fitting. Altogether, these studies highlight the role of SLN in determining muscle metabolic phenotype.

1.2.3. Sarcolipin, Fibre Type and Muscle Mass

In addition to shifting the metabolic profile of skeletal muscle, changes to $[Ca^{2+}]_{cyt}$ resulting from the interaction between SERCA and SLN may also affect muscle performance by altering muscle mass and the expression of the contractile proteins. With respect to the latter, calcineurin (Cn), a serine-threonine phosphatase activated specifically in response to low, sustained increases in $[Ca^{2+}]_{cyt}$, has been implicated in signalling for the expression of various myosin heavy chain (MHC) isoforms (69). When activated, Cn dephosphorylates nuclear factor activating T-cell (NFAT) proteins, which are a family of transcription factors containing five isoforms (NFATc1-c4 and NFAT5) (70). Under normal physiological conditions, NFATs are heavily phosphorylated and located in the cytoplasm but upon dephosphorylation by Cn, a nuclear localization signal is uncovered and NFATs translocate to the nucleus where they act as transcription factors (71, 72). Within skeletal muscle, isoforms NFATc1, c2, c3 and c4 are shown to activate the expression of MHC isoforms. Extensive research has been conducted in order to link specific NFAT isoforms to the corresponding MHC isoform, yet the relationship is complex and examples of both cooperation and antagonism between NFAT isoforms can be found in the literature (73-75). One finding that is consistent is the link between NFATc1 and the expression of slow MHC1 (75-78). Although studies have shown that there is cooperation between NFATc1-4 for MHC1 expression (75), constitutively active NFATc1 alone is enough to promote MHC1 expression (76). Furthermore, the expression of fast variants of MHC (llA, IIX, IIB) are independent of NFATc1 as the expression of these fast MHC isoforms in NFATc1 knockout cells is similar to that of control (75). Variation in stimulation patterns have been shown to differentially activate these NFAT isoforms wherein low sustain stimulus promotes

NFATc1 and MHCI, while higher, faster stimulus promotes NFATc3/c4 and MHC II variants (75). As SLN alters SERCA activity and promotes increases in $[Ca^{2+}]_{cyt,}$ SLN could alter muscle fibre type profile by activating Cn.

In addition to altering fibre type, Cn activity has been shown to play an important role in muscle hypertrophy and recovery from damage (79, 80). Activated Cn has been shown to promote muscle growth by activating myogenic transcription factors, myocyte enhancing factor 2 (MEF2) and MyoD (81), and has been implicated as both a negative regulator of myostatin (82) and an essential downstream component within the IGF-1 hypertrophy pathway (83, 84). In line with the notion that Cn is a muscle growth promotor, the administration of the Cn inhibitor, cyclosporine A, has been shown to prevent hypertrophy during both hindlimb unloading/reloading and mechanical overload models (85-88). However, it should be noted that not all research on the role of Cn in adaptive muscle growth is consistent, with the effects of Cn likely depending on the Ca^{2+} stimulus, isoform of Cn/NFAT, and the dose of Cn inhibitor administered (89). Finally, SLN may also modulate muscle mass via CAMKII. Activated CAMKII has been shown to localize within the nuclei where it directly phosphorylates transcriptional machinery including serum response factor (SRF) (90), myogenin (91), and histone deacetylase 4 (92) promoting skeletal muscle differentiation.

Interestingly, although it is clear that SLN alters SERCA function and $[Ca^{2+}]_{cyt}$ in murine muscle (53, 66), both $Sln^{-/-}$ and Sln^{OE} mouse muscles display no changes in either MHC isoform expression or muscle cross sectional area compared to WT (22, 64, 66). The similarities in muscle mass and fiber type between WT, $Sln^{-/-}$ and Sln^{OE} mice may seem surprising; however, this is consistent with previous research showing activated Cn alone does

not stimulate muscle adaptation, with mice overexpressing the overactive Cn mutant (CnA*) not showing any differences in basal muscle fiber type composition or size compared with wild type controls (93). Only when an added stimulus was applied, such as chronic muscle overloading, were effects of Cn activation seen with exaggerated muscle hypertrophy and accelerated fast-to-slow oxidative fiber type shifts. Similarly, the effects of SLN on Cn-mediated muscle plasticity may not be parsed out in conditions without an added stimulus (93). In fact, the relationship between Cn, SLN and the ensuing advantageous effects to muscle function were investigated by Fajardo et al (2017) using the mechanical overload model. Mechanically overloaded plantaris displayed ectopic SLN expression and elevated Cn activity, which resulted in muscle hypertrophy, increased fiber number, and a fast-to-slow fiber type shift. Likewise, the ablation of SLN improved SERCA activity but blunted advantageous hypertrophic signaling as $Sln^{-/-}$ overloaded plantaris myofibers failed to hypertrophy and transition to a slow-oxidative phenotype (66). Therefore, altogether, it seems that increases in SLN expression and Cn signaling do not alter the basal expression of MHC isoforms or muscle mass, but SLN does mediate fiber type shifts and hypertrophy via Cn in the face of overloading stimuli.

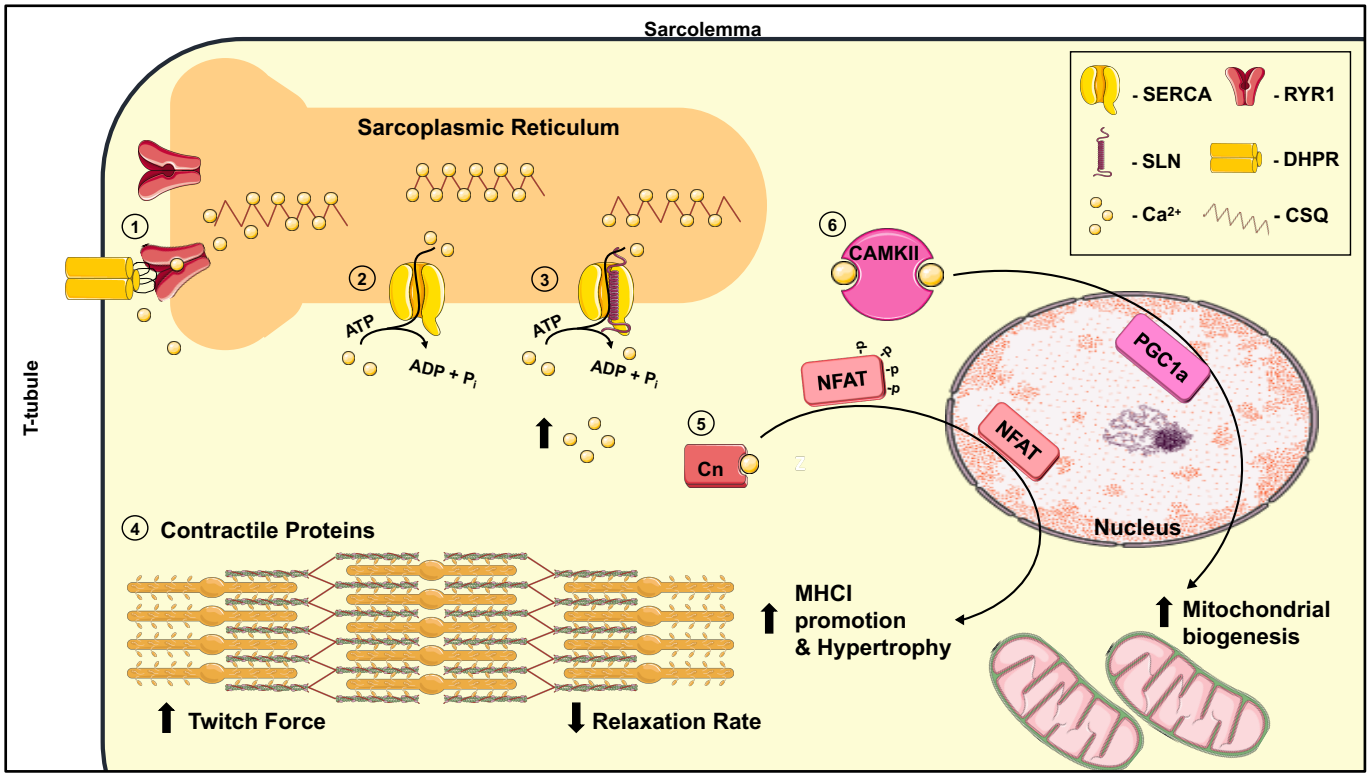


Figure 3. The Role of Sarcolipin in Muscle Function and Cell Signalling

1. Stimulated by an action potential, Ca^{2+} is rapidly released from the sarcoplasmic reticulum through RYR1 channels, where it binds to contractile filaments resulting in force production. 2. Muscle relaxation is facilitated by SERCA where, optimally, two Ca^{2+} ions are transported per one molecule of ATP hydrolyzed. 3. When SLN is bound to SERCA, it reduces the affinity and efficiency of the pump. 4. The resulting amplification of cytosolic Ca^{2+} increases twitch force and reduces relaxation rate. 5. SLN has also been linked to the promotion of Ca^{2+} signalling. Activated Cn dephosphorylates the transcription factor NFAT, resulting in its nuclear translocation and the promotion of both MHCI expression and hypertrophy. 6. Activated CAMKII promotes mitochondrial biogenesis via PCG1α.

1.3 Sarcolipin in Disease States

Interestingly, sarcolipin expression has also been shown to be dynamic in response to differing stress conditions. Several groups have found that SLN is upregulated in atrophying skeletal muscle. Tomiya et al (2019) found that following two-week cast immobilization, the lower limb muscles (soleus, plantaris and gastrocnemius) displayed significant atrophying along with increased SLN expression compared to the muscles of the contralateral limb (94). Similarly, a study by Braun et al (2021) found that following a 35-day spaceflight mission, where animals experienced unloading due to antigravity, SLN was significantly upregulated in mouse soleus muscles (95). Finally, in the functional overload study mentioning previously, Fajardo et al (2017) found a 14-fold increase in SLN expression in tenotomized soleus compared to control (66). Although it may intuitively seem that an increase in SLN and subsequent reduced SERCA activity would be detrimental in these instances, potentially adding to mitochondrial dysfunction and activating deleterious Ca^{2+} signalling pathways, this is not the case. In fact, when the soleus muscles of *Sln*$^{-/-}$ mice were tenotomized, it resulted in worsened atrophy, witnessed by reduced muscle weights and smaller muscle fibre cross sectional area, compared to the tenotomized WT soleus (66). Fajardo et al (2017) found the upregulation of SLN in unloaded soleus was accompanied with increased activation of Cn and a reduction in cytosolic phosphorylated NFAT, which was not witnessed in *Sln*$^{-/-}$ muscle (66). As such, the authors interpreted the increase in SLN as a compensatory mechanism, activating Cn and promoting the maintenance of muscle mass and slowing the fibre type transition commonly witnessed with unloading (66).

Similar to muscle atrophy, SLN content has also been shown to be upregulated in a plethora of skeletal muscle myopathies. More specifically, SLN has been shown to be upregulated in myotonic dystrophy (96, 97), centronuclear myopathy (CNM) (98, 99), Emery-Dreifuss dystrophy (100), and Duchenne muscular dystrophy (101-103). Within the myopathy literature, there has been debate about whether this dynamic increase in SLN content is compensatory or maladaptive in nature and the most contentious debate can be found in the literature surrounding Duchenne muscular dystrophy (DMD). DMD is an X-linked genetic neuromuscular disorder resulting from mutations and/or deletion of the gene *dystrophin* which results in alterations to the key cytoskeletal protein, dystrophin (104). The mutation or absence of dystrophin results in a destabilized plasma membrane causing generalized weakness and a flooding of the cytosol with cytotoxic levels of extracellular Ca^{2+} (105). While there is phenotypic variation due to differing genetic mutations, in human patients, DMD manifests as progressive muscle degeneration that ultimately results in loss of independent mobility and early fatality with a median life expectancy of 22 years (106, 107). Schneider et al (2013) were the first to report both ectopic and endogenous increases in SLN expression along with reductions in SERCA activity in muscle from *mdx* mice, the murine model of DMD (108). Schneider interpreted this increase in SLN content as a maladaptive feature of DMD speculating that reductions in SERCA activity might exacerbate the Ca^{2+} dysregulation common to this condition (108). Some groups also speculated that SLN was maladaptive given the upregulation of SLN was greater in DMD models with greater disease severity (102, 108). For instance, *mdx/utr*$^{-/-}$ mice, which lack both dystrophin and its functional homologue utrophin, display an even greater upregulation of SLN than *mdx* mice and a worsened disease phenotype which more closely mimics human DMD patients (108). Accordingly, Voit et al

(2017) found that *mdx/utr*$^{-/-}$ mice with either genetic SLN knockdown or knockout displayed normalized body weights, improved muscle pathology and contractility, along with an extended lifespan compared to their *mdx/utr*$^{-/-}$ counterparts which drastically overexpress SLN (102).

However, there is also evidence to suggest that SLN upregulation in DMD may be an advantageous adaptation. In contrast to the study by Voit et al (2017) (102), Fajardo et al (2018) found that *mdx* animals lacking SLN (*mdx/sln*$^{-/-}$) displayed a worsened disease phenotype than *mdx* animals, as indicated by decreased myofiber cross sectional area, reduced daily ambulation and increased serum creatine kinase (101). Additionally, the authors found that Cn signalling was blunted in the *mdx/sln*$^{-/-}$ soleus compared to the soleus of *mdx* animals (101). Activated Cn may help to improve DMD pathology by signalling for muscle growth and fibre type shifts as mentioned previously, but also by promoting the expression of utrophin. In the absence of dystrophin, utrophin aids in stabilizing the plasma membrane which reduces the influx of extracellular Ca^{2+}, lessening the strain on mitochondria and reducing the activation of cell death signalling (109). Indeed, Fajardo et al (2018) found that the reduction in Cn signalling in *mdx/sln*$^{-/-}$ muscle was accompanied by lower levels of utrophin content, signifying that the upregulation of SLN in *mdx* muscle is at least in part a compensatory mechanism to improve membrane stability. This Cn-utrophin pathway may help to explain some of the discrepancies between the findings of Voit et al (2017) and Fajardo et al (2018), as the use of the *mdx/utr*$^{-/-}$ model would eliminate one of the main benefits of increased SLN expression. In fact, Voit et al (2017) found that while either genetic knock down or full knock out of SLN improved muscle health in *mdx/utr*$^{-/-}$ animals, the haplodeficient SLN *mdx/utr*$^{-/-}$ animals who

had normalized SLN levels fared better than *mdx/utr*$^{-/-}$ animals with full genetic deletion of SLN with respect to lifespan and DMD pathology (102). This suggests that even when the SLN-Cn-Utr pathway is blocked, the expression of some SLN is still beneficial in improving DMD disease outcomes.

This phenomenon can also be witnessed in other disease models, in which utrophin does not play an essential role. Similar to DMD, SLN expression has also been shown to be elevated within muscle from mouse models for centronuclear myopathy (CNM) (99, 110). While the underlying genetic mutations for CNM vary, this grouping of inheritable neuromuscular disorders are characterized by three main histological features: centrally located nuclei, a central aggregation of oxidative activity and an increased proportion of type I muscle fibres (~90% of total fibres). With respect to CNM pathology, there is fibre type specificity wherein the central localization of nuclei and oxidative activity along with general atrophying predominantly occurs to type I fibres. Given that the Cn-NFAT signalling pathway is primarily responsible for type I fibre expression, one would hypothesize that increased SLN would be detrimental as it would increase cytosolic Ca^{2+} activating Cn, further promoting the shift in fibre type, and exacerbating CNM pathology. However, Fajardo et al (2017) found no differences in the fibre type profile or the presence of CNM disease markers with genetic SLN ablation in CNM muscle (98). In fact, SLN deletion resulted in further reductions to muscle weight, lower total myofiber counts and smaller cross-sectional area of type II fibres compared to the CNM counterparts which upregulate SLN (98). Similar to the findings in *mdx* animals, deletion of SLN in this CNM model resulted in blunted Cn signaling as witnessed by an increase in the ratio of phosphorylated NFAT to total NFAT. While it is unclear why the

reduction in Cn-NFAT signaling with SLN ablation did not impact muscle fibre type within this model, these findings suggest that the upregulation of SLN in CNM promoted muscle maintenance and hypertrophy specifically within type IIa fibres.

1.4 SERCA Protection

In addition to its role in promoting utrophin expression, shifting fibre type and promoting muscle growth, recent research has suggested SLN may play a role in protecting SERCA from oxidative damage. Within muscle, it is well established that reactive oxygen/nitrogen species (ROS/RNS/RONS) play a significant role in modulating Ca^{2+} cycling by targeting both RYR and SERCA (111, 112). While there is variation between isoforms, the amino acid sequencing and structure of SERCA make it especially susceptible to posttranslational modification by RONS. SERCA1a and SERCA2a contain 24 and 26 Cys residues, respectively, which are vulnerable to oxidation, nitrosylation, or S-glutathiolation by RONS (113-115). Specifically, the integral M4-M5 loop, which forms the bulk of the cytosolic head piece of SERCA, contains Cys thiols located at key positions near the phosphorylation site (Cys344 and Cys349), the nucleotide binding pocket (Cys525) and the hinge region (Cys674 and Cys675), which, when oxidized, can alter the catalytic function of SERCA (116, 117). Additionally, SERCA2a is also uniquely liable to Tyr nitration (Tyr294, Tyr295, and Tyr753) with oxidative stress (111, 118). While it was originally thought that RONS were solely detrimental to cell health, it is now clear these highly reactive molecules, when produced at low levels, can act as regulatory agents for cell function and signalling. Indeed, treatment with low concentrations of RONS has been shown to increase SERCA activity and SR Ca^{2+} uptake likely via S-glutathiolation of Cys674 (119, 120). Conversely, when exposed to higher concentrations of RONS SERCA activity is

inactivated. The seminal papers by Viner et al (1996 and 1999) displayed clearly a dose dependent relationship, in that treatment with lower concentrations of peroxynitrate resulted in reversible oxidation of several M4-M5 cys thiols and reduced SERCA activity, while higher concentration treatments caused irreversible SERCA oxidation and loss of activity likely involving the oxidation of additional amino acid residues (115, 121).

In times of cell stress, mitochondrial overload and the infiltration of inflammatory cells can sway the redox balance resulting in elevated RONS levels. Several stress conditions, such as atrophic unloading (95), aging (116, 122, 123) and various myopathies (124-126), display elevations in ROS/RNS production and reduced SERCA activity. Given that RONS mediated SERCA damage would result in elevated cytosolic [Ca^{2+}] potentially exacerbating mitochondrial overload and activating cell death signalling, strategies to protect SERCA from oxidative damage and improve SR Ca^{2+} uptake are of paramount importance. Treatment with antioxidants have been shown to improve SERCA activity and muscle function in models of aging (127), DMD (128) and malignant hypothermia (129). Similarly, research by Tupling et al (2004) found that the chaperone, heat shock protein 70 (HSP70), prevented thermal inactivation of SERCA1a *in vitro* by binding and stabilizing the nucleotide binding domain (130). This protective effect of HSP70 may be even more pronounced for SERCA2a. In fact, the co-transfection of SERCA2a and HSP70 in HEK-293 cells not only prevented thermal inactivation but resulted in increased maximal SERCA activity in heat stressed cells compared to control cells (131). Likewise, increased expression of HSP70 either via transgenic overexpression or pharmacological induction, in mouse models for DMD has been shown to increase SERCA activity, improve muscle pathology and function and reduce serum creatine

kinase (124). More recently, research has begun to examine whether PLN or SLN may act in a similar manner to HSPs as SERCA protective proteins. Fu et al (2020) found that both SLN and PLN had the capacity to protect SERCA2a function in the face of heat stress by preventing oxidative damage as co-expression of either SLN or PLN with SERCA2a prevented carbonylation and nitrosylation of the pump (132). This finding translated to animal models where the transgenic ablation of either PLN or SLN resulted in greater reductions in maximal SERCA activity with heat stress compared to WT (132), suggesting that even endogenous expression of PLN and SLN in WT muscles preserves SERCA function in times of cell stress. Indeed, the protective nature of SLN specifically seems fitting given the dynamic upregulation during unloading and muscle disease mentioned previously.

1.5 A Potential Role for SLN in Aging

There are several commonalities between skeletal muscle disease and aging, making the role of SLN in aging an interesting avenue for research. It is well established that reductions in muscle mass, performance and muscle quality are witnessed with aging, collectively coined sarcopenia (133). With respect to muscle function, reductions in maximal isometric force in older adults have been found in both upper and lower body muscle groups starting roughly in the fifth decade of life (134, 135). In addition to reduced maximal isometric force, muscle power, a measure of both speed and force production which more directly reflects everyday functional ability, has been shown to decline even more rapidly than strength with aging (136, 137). Finally, both the rate of contraction and relaxation are generally slowed in muscle from older individuals (138, 139).

A portion of the age-related loss in muscle performance can certainly be attributed to reductions in overall muscle mass. Longitudinal studies of older adults (~75y) have found an annual rate of muscle loss of 0.64–0.7%/y in females and 0.8–0.98%/y in males, albeit these reductions vary and are confounded by factors such as race and physical activity levels (140, 141). At a cellular level, this age related muscle loss is attributed mainly to selective loss and atrophy of type II fibres, creating an apparent shift towards smaller type I fibres (134). In conjunction with muscle atrophy, the decline of muscle performance may also be due to a decline in the number of motor units and reductions in muscle quality with the infiltration of fat and connective tissue witnessed in aging muscle (15, 142, 143). It should, however, be noted that age related reductions in muscle mass do not entirely account for the loss of muscle performance with aging. A five-year longitudinal study of over 1500 participants by Delmonico et. al (2009) found that the decline in muscle strength is more rapid than the concomitant loss in muscle mass, and that those who did not lose muscle mass still displayed reductions in muscle strength (144). In conjunction with the findings of Delmonico et al., reductions in specific force (force per cross sectional area) can still be witnessed in both intact and mechanically skinned single muscle fibres from older subjects compared to their younger counterparts (139, 145, 146). The loss of muscle function in excess of or in absence of losses in muscle mass suggest that there are additional factors at the myofibre level beyond atrophy which contribute to the loss in muscle function with aging.

One such cellular factor implicated in loss of muscle function with aging is dysregulation of Ca^{2+} homeostasis. Alterations to both resting and peak transient $[Ca^{2+}]_{cyt}$ can be witnessed within aging muscle. At rest, $[Ca^{2+}]_{cyt}$ has been shown to be increased in both

slow and fast twitch fibres of older adult muscle compared to healthy younger counterparts (147). While elevations to $[Ca^{2+}]_{cyt}$ are essential for contraction, sustained increases in resting $[Ca^{2+}]_{cyt}$ may reduce muscle function by altering the sarcolemmal membrane potential and by activating proteolytic and apoptotic signalling pathways (148). Accordingly, studies have found aging muscle displays a more negative mechanical threshold, signifying reduced muscle contractility, as well as increased calpain activity and caspase activation (147, 149, 150). Converse to the rise in resting $[Ca^{2+}]_{cyt,}$ peak intracellular Ca^{2+} transients have been shown to be reduced by nearly 50% in both older mouse and human muscle (151, 152). This significant reduction in cytosolic free Ca^{2+} available to bind to contractile proteins may account for reductions in force output seen in older adult muscle and efforts to restore Ca^{2+} dynamics has been shown to improve muscle performance (147, 153).

The etiology of disrupted Ca^{2+} dynamics in aging skeletal muscle is likely multifaceted, including structural alterations and changes to both content and function of Ca^{2+} handling proteins. Both the SR and the calcium release unit, comprised of the junctional domains of the SR and both adjacent T-tubules, undergo structural alterations with aging which may impact Ca^{2+} homeostasis (154, 155). Morphological changes witnessed with aging include the presence of elongated SR tubular aggregates, increased variability in the localization and quantity of CRUs along a muscle fibre, and greater frequency of dyads (junctions formed between a T-tubule and a single SR terminal cisternae (154, 155). These changes to CRU structure are accompanied by a reduction in the content of DHPRα1 subunit with aging (156). As a result, functional uncoupling of DHPR and RYR1 is witnessed with aging, ultimately reducing voltage induced SR Ca^{2+} release (157). Similarly, SR Ca^{2+} uptake is also impacted

with aging and likely contributes to altered Ca^{2+} homeostasis. Fluctuations in SERCA content with aging are controversial with some researchers finding no changes in SERCA content with aging (118), while others found a reduction in SERCA1a and no change in SERCA2a, mimicking the fibre type shifts in aging (158). While changes to SERCA content are unclear, a reduction in SERCA ATPase activity is consistently witnessed in aging muscle (118, 138, 158, 159), with most studies finding an accompanying reduction in Ca^{2+} uptake (118, 138).

Oxidative stress, a hallmark characteristic of aging muscle (151), is also likely to impact the function of Ca^{2+} handling proteins and $[Ca^{2+}]_{cyt}$ with aging. While studies suggest there is no change in total RYR1 content in aging muscle (158), RYR1 displays increased oxidation and nitrosylation accompanied by reductions in the RYR1-stabilizing protein calstabin-1 (153). Oxidation in this manner results in "leaky" RYR1 channels which may be responsible not only for the increase in resting $[Ca^{2+}]_{cyt,}$ but also for the reductions in peak Ca^{2+} transient as it may deplete SR Ca^{2+} stores. Similar to RYR1, reductions in SERCA activity may be due in part to posttranslational modification by RONS. In fact, Viner et al. 1999 found that with aging there was increased nitrosylation of Tyr resides in the M4-M9 transmembrane domains, likely affecting SERCA function due to the proximity to the Ca^{2+} binding sites (118). While additional factors, such as denervation, DHPR-RYR uncoupling and the expression of respective regulatory proteins are likely to impact RYR1 and SERCA function with aging aside from oxidative damage, efforts to increase the activity of RYR1 and SERCA have proven to be fruitful for improving Ca^{2+} dynamics and muscle function. In a study by Andersson et al (2011), treatment with S107, a pharmacological RYR1 stabilizing agent, improved RYR1-calstablin-1 binding, increased tetanic SR Ca^{2+} release and improved force generation in older

animals (153). Likewise, Qaisar et al (2019) found that treatment with CDN1163, an allosteric activator of SERCA, improved SERCA activity and muscle force production in an animal model for oxidative stress (160). A subsequent study by Qaisar et al (2020) demonstrated that improving SERCA function had similar results in aging, where CDN1163 treatment not only improved SERCA function but also restored muscle mass and force generation to that of younger animals (122). Collectively these studies suggest that restoring Ca^{2+} dynamics within aging muscle offers a viable therapeutic method to improve not only muscle function but also muscle mass maintenance.

In light of this, it is surprising that few studies have examined how the SERCA regulatory proteins are impacted with aging. Of the SERCA inhibitory proteins, the majority of literature in aging has been focused on the role of PLN in aging cardiac muscle. While its well established that PLN plays a role in cardiomyopathy and heart failure, studies have found that there is no change in PLN content in otherwise healthy aging hearts (161, 162). Further, it seems that improvements in SERCA function observed with PLN ablation are maintained throughout the aging process (161). Conversely, the studies examining SLN content within skeletal muscle have found alterations with aging. To date, two studies have examined SLN content in aging skeletal muscle, both finding that SLN content increased when compared to younger counterparts (163, 164). Lin et al (2018) examined changes to gene expression in rectus femoris muscle from three and 24 month old mice and found that there was over a two-fold increase in *Sln* mRNA expression in older muscle compared to younger (163). Furthermore, Qaisar et al (2018) assessed both SLN content and SERCA function with aging and, in accordance with the literature, SERCA activity was reduced with aging which was

accompanied by an increase in SLN content (164). The authors hypothesized that the reductions in SERCA activity may be attributable in part to the increase in SLN (164), however there have yet to be any studies examining how SLN ablation alters SERCA function with aging and secondarily how this may affect downstream factors such as metabolism, muscle mass or contractile function. While it is very possible that the increase in SLN does inhibit SERCA activity, the evidence from atrophic and myopathy models suggests that this increase in SLN expression may also be a compensatory effort to alter Ca^{2+} signalling and protect SERCA from oxidative stress. As such this thesis seeks to examine the role of SLN in skeletal muscle with aging with respect to muscle metabolism, mass and function.

1.6 SLN and Sex Differences in Skeletal Muscle

In addition to examining the role of SLN within aging muscle, this thesis will also seek to discern if there are significant differences in SLN content and function between biological sexes. While it is yet to be determined if SLN content or function varies across sexes, there are several areas which are impacted by both SLN and biological sex, including body composition, muscle fuel utilization and performance. With respect to body composition, healthy adult males generally have greater body mass with a lower body fat percentage and greater lean body mass than their female counterparts (165). The greater overall muscle mass of males results in greater absolute force production, however there are no differences in maximal force between sexes when normalized to muscle mass (166). At the myofiber level, females have also been shown to have greater resting intramyocellular lipid (IMCL) content compared to males when matched for VO_{2peak} per fat free mass (167). In addition to having greater fat stores than males, females have also been shown to have a greater reliance on fats as a fuel source. Several studies

have found females have a lower respiratory exchange ratio (RER) during endurance exercise, indicative of a greater reliance on lipids and relatively lesser reliance on carbohydrates as a fuel source compared to males (168-174). The increased fat stores and greater reliance on fat as a fuel source is thought to contribute to enhanced fatigue resistance as females have been shown to exhibit less fatigue during single and repeated sprints as well as ultra-marathon distances (175-177). While there are likely additional factors at play, this can also be witnessed during isometric exercise where females demonstrate fatigue resistance compared to males given the same relative intensity (178-181). Finally, aligned with the disparities in fatigue resistance and fuel utilization, studies examining human muscle biopsies find that females display an increased proportion of type I fibres and less type IIA and IIX fibres than males (182, 183).

There is extensive research showing the above sexual dimorphism can be attributed, at least in part, to variations in sex hormones between males and females. Testosterone induces skeletal muscle adaptations by binding to androgen receptors that can regulate gene transcription and supplementation with testosterone has been shown to increase protein synthesis and muscle mass in both postmenopausal females and hypogonadal males (184, 185). Conversely, studies in which male rats were supplemented with estradiol have found both increased resting IMCL and lessened muscle damage post exercise signifying improved fatigability (186, 187). Furthermore, estrogen also seems to play a role in fuel selection, as human males display lower RER values during endurance exercise when supplemented with estrogen than placebo (188).

Alterations in the concentration of these sex hormones differs significantly throughout the lifespan. Briefly, post-puberty males have relatively stable low levels of progesterone, estrogen and high levels of testosterone, peaking around 20yrs old at roughly 15nmol/L and declining slightly with aging (189, 190). Conversely, while females have consistently lower testosterone levels (<1nmol/L), after puberty they undergo menstrual cycles throughout the reproductive lifespan which see large variations in several sex hormones, including follicle-stimulating hormone, estrogen, luteinizing hormone, and progesterone. For a review of the female menstrual cycle and the accompanying interplay of sex hormone fluctuations, see Reed & Carr (2018) (191). In contrast to the relatively slow and small decline in testosterone in aging males, females undergo the process of menopause in which they experience the cessation of their menstrual cycle and a drastic decline in estrogen levels. While the stark decline in estrogen in females during menopause is clearly linked to reductions in bone density and more generally associated with changes in body composition (192, 193), studies examining muscle mass and strength in post-menopausal females to do not find increases in the loss of muscle mass or strength in excess of their aging male counterparts, suggesting that additional factors outside sex hormones may contribute to changes in muscle mass and function with aging. In a scoping review of over 5000 articles by Haynes et al (2020), the rate of loss in lower body isometric strength was nearly identical between aging males and females with a rapid decline in isometric strength occurring in the late 50s (194). Furthermore, when examining dynamic isokinetic lower body strength, a measure more indicative of physical ability, the authors found that males displayed greater rates of decline in strength especially in later life (194). However, it must be noted that despite the increased rate of loss, males maintain greater absolute muscle mass and strength than females throughout the lifespan. Accordingly, females have been found

to be at greater risk of both frailty and sarcopenia which is especially important as females have an extended life expectancy compared to males (195, 196).

While changes to sex hormones certainly impact muscle function, mass and metabolism throughout the lifespan, disparities in SLN content could also exacerbate the differences between males and females. In young adults, the literature to date has exclusively examined SLN content and function in male animals (22, 34, 52, 57, 66), however, there are several reasons to hypothesize that females may express greater SLN content than males. Studies in both animals and humans have reported that males display faster maximal rates of relaxation suggesting greater SERCA activity (197-199), and while these discrepancies in relaxation rate between males and females may be attributable to the differing fibre type profiles, disparities in SLN content could aid in explaining the delayed relaxation rates in females. Moreover, SLN could play a role in the sexual dimorphism in fibre type as it has been shown to activate Cn signalling and type I fibre promotion, albeit this seems to be distinct to overload and stress conditions. Furthermore, given the findings from developing muscle which suggest SLN promotes mitochondrial biogenesis and lipid oxidation via Ca^{2+} signalling (64), SLN could play a role in promoting this metabolic profile in female muscle. Finally, if greater SLN content in females persisted throughout aging, it may aid in protecting SERCA from age-induced oxidative stress, aligning with previous studies showing that males display greater age-related oxidative damage than their female counterparts (200-203). In order to better understand the relationship between SLN and biological sex, this thesis examined SLN content in both sexes at an adult and older adult age group. Further, this thesis also sought to examine

how potential sex differences in SLN content may affect muscle metabolism, and the protection and maintenance of SERCA function and muscle mass with aging.

Chapter 2: Overarching Purpose and Thesis Objectives

2.1 Overarching Purpose

Previous research has shown that through its interaction with SERCA, SLN has four distinct impacts on muscle: 1) SLN increases twitch force and slows relaxation 2) increases energy expenditure associated with SR Ca^{2+} uptake, 3) protects SERCA function in the face of RONS 4) and activates Ca^{2+} signalling promoting muscle mass, shifts to fibre type and changes in the metabolic properties of muscle. The overarching purpose of this thesis was to examine how SLN content varies between sexes in adult and aged muscle and to discern the role of SLN in regulating energy expenditure, protecting SERCA and promoting muscle mass and function with aging in both male and female mice.

2.2 Study 1 - Examining the Impact of SLN ablation on SERCA Function and Whole-Body Energy Expenditure between Sexes.

2.2.1 Objectives & Hypotheses

The main objectives of Study 1 (Chapter 3) were:

- To elucidate if sex differences exist in the expression of SLN
    - Hypothesis: SLN expression will be greater in soleus muscles of WT females than their male counterparts.
- To discern how SLN ablation impacts SERCA efficiency, whole body metabolic expenditure and adiposity in both male and female mice.

- Hypotheses: SLN ablation will result in greater Ca^{2+} uptake to ATPase ratios, signifying greater SERCA efficiency than WT muscle, in both sexes. As it is hypothesized that WT females will display greater SLN content than WT males, the impact of SLN ablation will be more evident between WT and *Sln*$^{-/-}$ females than male groups. Accordingly, female *Sln*$^{-/-}$ mice will display reduced whole body energy expenditure and greater adiposity specifically compared to WT females, while males may not display differences between genotypes. In accordance with the literature, it is hypothesized that males will display greater body weights, higher absolute whole body energy expenditure, and lower adiposity than females, regardless of genotype.

2.3 Study 2 - The Role of SLN in SERCA Protection with Aging in Male and Female Mice

2.3.1 Objectives & Hypotheses

The main objectives of Study 2 (Chapter 4) were:

- To examine the effects of aging on the expression of SLN and SERCAs in both males and females
    - Hypotheses: SLN content will be greater in females and older animals. The expression of SERCA isoforms will generally follow the known trends in fibre type differences with sex and aging, where males will display greater SERCA1a than females and females more SERCA2a than males. Compared to young animals, older adults will display increases to SERCA2a regardless of sex.

- To examine the effects of SLN ablation on SERCA activity with aging in both males and females
    - Hypotheses: In young adults, WT males will display greater SERCA function than WT females and SLN ablation will improve SERCA function with no differences between male and female $Sln^{-/-}$ (4-6M: $Sln^{-/-}$ males = $Sln^{-/-}$ females > WT males > WT females). Aging will significantly reduce SERCA function, and animals who display low SLN (WT males) or no SLN ($Sln^{-/-}$ animals) will display greater reductions to SERCA function with aging (18M+: WT females > WT males > $Sln^{-/-}$ males = $Sln^{-/-}$ females).
- To discern if SLN is protective against age related SERCA oxidative damage in both males and females
    - Hypotheses: – In young adult animals there will be no statistically significant differences in oxidative damage between sex or genotype groups. Aging will increase oxidative damage to SERCA in all animals, with animals that display relatively lower SLN (WT males) and no SLN ($Sln^{-/-}$ animals) displaying greater damage (18+ M: $Sln^{-/-}$ males = $Sln^{-/-}$ females > WT males > WT females).

2.4 Study 3 - The Impact of SLN Ablation on Cn Signaling and Skeletal Muscle Phenotype and Function with Aging and Sex

2.4.1 Objectives & Hypotheses

The main objectives of Study 3 (Chapter 5) were:

- To examine the effects of age and SLN ablation on Cn signalling in both males and females.
    - Hypotheses: In young adult animals, sex and genotype will not impact Cn signalling. Aging will increase Cn signalling, with greater increases in animals with greater SLN content. As it is hypothesized that WT females will display greater SLN content than SLN males, in all age groups, older adult WT females will display the highest level of Cn activation (18+M: WT females > WT males > $Sln^{-/-}$ males = $Sln^{-/-}$ females).
- To discern how SLN ablation impacts muscle function, fibre type and muscle mass with aging in both males and females.
    - Hypotheses: In young adult animals, SLN ablation will result in faster rates of relaxation with no impact on maximal muscle force, muscle mass or fibre type in both males and females. In both age groups, males will display greater body and muscle mass, as well as greater absolute force than female counterparts, regardless of genotype. Aging will result in reduced muscle mass and force production, along with an apparent shift to type I fibre predominance compared to younger counterparts. In older adults, SLN ablation will result in further

reductions to muscle mass, blunted fibre type shifts and reduced muscle function compared to age matched WT animals. As aged WT females will display greater SLN content than WT males, the impacts of SLN ablation will be witnessed to a greater extent between older adult WT females and $Sln^{-/-}$ females than the age-matched male pairing.

Chapter 3: Examining the Impact of SLN ablation on SERCA Function and Whole-Body Energy Expenditure between Sexes.

3.1 Abstract

The sarco(endo)plasmic reticulum ATPase (SERCA) pump facilitates Ca^{2+} transport into the sarcoplasmic reticulum (SR), making the pump a primary regulator of Ca^{2+} homeostasis and skeletal muscle relaxation. SERCA activity is regulated by changes to intracellular [Ca^{2+}] and allosteric protein inhibitors, like sarcolipin (SLN). SLN binds to the transmembrane domain of SERCA, reducing SERCA Ca^{2+} affinity and uncoupling SERCA-mediated Ca^{2+} transport from ATP hydrolysis. Inhibition of SERCA by SLN ultimately results in delayed relaxation rates, increased energy consumption and heat production. As such, SLN expression has been implicated in protection against both diet-induced obesity and cold exposure. Previous research examining SLN in murine models has exclusively reported on male mice, leaving a gap in the literature surrounding the role of SLN in regulating SERCA function and energy expenditure in females. This study sought to address this sex bias in the literature by examining sex differences in SLN expression and investigating the impact of SLN ablation on SERCA function and energy expenditure in both male and female mice. Here, in wild type (WT) mice, it was found that SLN expression was greater in the soleus muscles of female mice than males. This was accompanied by greater SERCA2a expression in females and no differences in SERCA1a expression between sexes. SLN ablation was found to increase SERCA coupling ratio, as witnessed by greater Ca^{2+} uptake with similar ATPase activity in $Sln^{-/-}$ muscle compared with WT for a given [Ca^{2+}]. Pairwise comparisons revealed that $Sln^{-/-}$ females displayed a significantly greater coupling ratio than WT females, while the differences between

male genotype groups did not reach statistical significance, suggesting that SLN ablation impacted females to a greater extent than males. When examining the anthropometric and metabolic data, female mice, regardless of genotype, displayed greater adiposity along with lower body weight, food intake, absolute VO_2, and RER than their male counterparts. This study hypothesized that SLN ablation would reduce energy expenditure to a greater extend in females than males, as SLN content would be greater in WT females. Converse to this hypothesis, the current study found a sex by genotype interaction wherein male $Sln^{-/-}$ mice displayed a greater reduction in absolute VO_2 compared to WT males than that witnessed between female groups. When VO_2 was normalized to body weight, the sex by genotype interaction was eliminated and a main effect of sex remained, with male mice displaying lower relative VO_2 than females. While the impact of sex was contrary to the hypotheses, SLN ablation significantly reduced whole-body energy expenditure as expected. In fact, $Sln^{-/-}$ mice displayed lower VO_2 than WT for all measures of absolute and relative VO_2 in both sexes. Accordingly, this study also found a main effect of SLN expression on adiposity levels despite similar habitual food intake. Here, the ablation of SLN seemed to have a greater impact in females, with pairwise comparisons revealing statistically greater adiposity in $Sln^{-/-}$ compared to WT females, which did not reach significance between male groups. In summary, this study confirmed the role of SLN in reducing SERCA efficiency in both sexes and reported the novel findings that females display greater SLN expression which was accompanied by a greater increase in the apparent coupling ratio of SERCA and adiposity upon SLN ablation. Future research should continue to build on the literature examining SLN expression and function in females, with special focus on conditions which display both sex differences and metabolic dysfunction, including type II diabetes, liver and kidney disease and sarcopenic obesity.

3.2 Introduction

The sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) pump is responsible for transporting Ca^{2+} from the cytosol into the lumen of the SR and is essential in eliciting muscle relaxation and maintaining Ca^{2+} homeostasis in skeletal muscle. While SERCA activity is primarily regulated by changes in cytosolic [Ca^{2+}], several small protein inhibitors have been discovered, including sarcolipin (SLN), phospholamban, (PLN), myoregulin (MLN) and neuronatin (NNAT). All of the aforementioned SERCA inhibitors, work to reduce the Ca^{2+} affinity of SERCA allosterically by binding to the transmembrane region of SERCA (18, 20, 21, 204). In addition to altering Ca^{2+} affinity, SLN also reduces SERCA efficiency by uncoupling SERCA Ca^{2+} transport from ATP hydrolyzation (45). Thus, the presence of SLN results in greater SERCA ATP hydrolysis to transport similar quantities of Ca^{2+}, leading to increased heat production and energy expenditure (22, 46).

Concerning thermogenesis, several research groups have examined SLN for its ability to promote heat production and provide protection against cold exposure (46, 52, 62). Genetic ablation of SLN in mice results in enhanced cold sensitivity witnessed by an inability to maintain core body temperature when exposed to acute cold at 4°C (52). Several studies have also found SLN expression is dynamically upregulated in response to bouts of cold exposure (68, 205, 206). Similarly, SLN upregulation has been witnessed in developing muscle, where it is speculated to offer thermal protection to neonatal mice, who are especially susceptible to hypothermia (27, 68). Post-natal cold exposure has been shown to prevent the downregulation of SLN witnessed with maturation, suggesting the developmental upregulation of SLN is, at least in part, thermogenic in nature (68).

While the upregulation of SLN with development likely offers protection against cold exposure, SLN also appears to play a role in adaptive metabolic remodeling of muscle during development. Research examining SLN ablation in developing muscle have found abnormal mitochondrial structure, reductions to mitochondrial DNA and fatty acid oxidation (64). Subsequently, several research groups have uncovered evidence implicating SLN in the promotion of oxidative metabolism and mitochondrial biogenesis outside of the context of development. Specifically, Maurya et al (2018) found that upregulation of SLN in myotubes triggered mitochondrial biogenesis by raising $[Ca^{2+}]_{cyt}$ levels and activating the transcriptional regulators, CAMKII and PGC-1α (64). Furthermore, transgenic overexpression of SLN in mice results in significant improvements in treadmill exercise endurance, enhanced fatigue resistance in isolated soleus and extensor digitorum longus (EDL) muscles, and elevated fatty acid metabolism compared to WT controls (49, 64). Recent work by Mengeste et al (2022) was the first to investigate the role of SLN in metabolic remodeling in human skeletal muscle by examining the effect of SLN knockdown in primary culture of human skeletal muscle (207). Here, SLN knockdown myotubes displayed diminished fatty acid oxidation, reduced PGC-1α gene expression and increased cellular lipid accumulation compared to control cells (207). Collectively, these studies suggest that SLN has the capacity to promote fatty acid oxidation and mitochondrial biogenesis by elevating $[Ca^{2+}]_{cyt}$ and activating PGC-1α.

It is important to note that in addition to promoting Ca^{2+} signaling for metabolic remodeling, SLN also directly impacts muscle energy expenditure through its ability to alter the stoichiometry of SERCA and increase the amount of ATP hydrolysis required to pump Ca^{2+} into the SR lumen (22, 46). Ablation of SLN in mice has been shown to reduce the

relative contribution of SERCA activity to resting muscle energy expenditure by 13% compared to WT mice (56), albeit this was not accompanied by significant differences in body weight or daily whole body VO_2 between *Sln*$^{-/-}$ and WT male mice (48). However, *Sln*$^{-/-}$ mice have been shown to expend less energy throughout submaximal treadmill exercise than WT counterparts, suggesting the impact of SLN on energy expenditure is greater during bouts of activity (57). Similarly, research examining the role of SLN in diet induced obesity has highlighted the impact of SLN expression on adaptive thermogenesis and whole-body energy expenditure. SLN expression in WT mice has been shown to be dynamically upregulated in response to the caloric stress of high-fat feeding (57), a time when excess energy expenditure is advantageous. The dynamic increase in SLN content in response to high-fat feeding is thought to occur to promote futile SERCA activity and increase energy expenditure, as high-fat fed *Sln*$^{-/-}$mice are more susceptible to diet induced obesity (52, 54), and display lower relative VO_2 during active periods than high-fad fed WT counterparts (57). Collectively, the literature suggests that while SLN ablation may not affect whole-body energy expenditure in chow fed male mice, SLN does alter whole-body energy expenditure under conditions where SERCA activity is elevated or the ratio of SLN:SERCA increases.

A notable gap in the current literature examining SLN with respect to energy expenditure and metabolism is the limited exploration in females. Previous research has predominantly focused on male mice, with no reports of SLN expression in female mice or examinations of potential sex differences between males and females. This is especially relevant given there are several areas in which both sex and SLN expression are significant affecting factors, including muscle performance, substrate utilization and fibre type profile.

Concerning muscle performance and fibre type, studies in both animals and humans have reported that females display slower maximal rates of relaxation and greater resistance to muscle fatigue than males counterparts (175-177, 197-199). The literature generally attributes the disparity in relaxation rate to sexual dimorphism in fibre type profiles, with males displaying a greater proportion of fast-twitch type II fibres than females (197, 199). However, SLN plays a direct role in slowing relaxation through the inhibition of SERCA, thus differences in SLN content between sexes could contribute to the sexual dimorphism in relaxation rate. Furthermore, if disparities in SLN expression exist between males and females, it may also impact fibre type, as SLN has been implicated in activating calcineurin (Cn) and promoting MHC1 expression (66). Similarly, SLN expression may be linked to fatigability. As previously mentioned, greater SLN expression in murine models has been shown to promote fatigue resistance through the activation of PGC1-a signalling and resulting increases in mitochondrial biogenesis and lipid oxidation (49, 53). This is especially interesting, given the well-established differences in substrate utilization between sexes. Numerous studies have found females display a lower respiratory exchange ratio (RER) during endurance exercise, indicative of a greater reliance on lipids and relatively lesser reliance on carbohydrates as a fuel source compared to males (168-174). The sexual dimorphism in muscle performance, substrate utilization and fibre type have been shown to be driven by variations in sex hormones between male and females, and throughout the female menstrual cycle. While the relationship between variation in sex hormones and SLN expression has yet to be examined, greater SLN expression in females could also contribute to the witnessed sex differences in relaxation rates, lipid oxidation and fatigue resistance. Investigating the sex-dependent expression and function of SLN may offer further insights into the regulation of muscle function and energy

expenditure between sexes. To address this gap in the literature, this study sought to examine potential sex differences in the content and function of SLN by examining SLN expression, measures of SERCA function and whole-body energy expenditure in male and female, WT and *Sln*$^{-/-}$ mice. The impact of this research has broad applications and may provide valuable insight into therapeutic targets for conditions involving both sex differences and metabolic dysfunction, including obesity, metabolic disorders and aging (168-177, 197-199).

3.3 Methods

3.3.1 Animal Model

The generation of a murine line with genetic ablation of SLN was described previously by Babu et al. (2007) (208). This chimeric/C57BL/6 *Sln*$^{-/-}$ mouse line was subsequently established as a continuous breeding colony at the Central Animal Facility of the University of Waterloo. The breeding of two heterozygous *Sln*-null animals resulted in the offspring of wild-type (+/+; WT), heterozygous (+/-), and homozygous (-/-) mice. To distinguish the genotype, all animals were ear notched, and RT-PCR was conducted using DNA extracted from the ear notches. For RT-PCR, the sample DNA was added to GB-AMP™ 2x PCR Master Mix (Genebio Systems, Inc.) along with 5' and 3' primers for both WT and *Sln*-null (SLN-WT, forward, 5'-TGT CCT CAT CAC CGT TCT CCT-3'and reverse 5'-GCT GGA GCA TCT TGG CTA ATC-3'; *Sln*-null, forward, 5'- GTG GCC AGA GCT TTC CAA TA-3'and reverse 5'-CAA AAC CAA ATT AAG GGC CA-3'). Samples were then placed in a thermal cycler (MJ MINI, Bio-Rad, Canada) and denatured for 3 min at 94°C, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 54°C, and extension for 60 s at 72°C, followed by a final extension at 72°C for 7 min. The amplified products were then separated on a 1% agarose gel containing 0.01% ethidium bromide (BioShop, Canada) and identified using a ChemiDoc imaging system (Bio-Rad, Canada). For this study, experiments were conducted on littermate WT and *Sln*$^{-/-}$ mice, both male and female, aged 4-6 months. All animals were housed in an environmentally controlled room with a standard 12:12 light/dark cycle and allowed access to food (Tekland 22/5 Rodent Diet, Harland-Tekland, Madison, WI) and water ad libitum. The study was approved by the Animal Care Committee of the University

of Waterloo, and all procedures were performed in accordance with the Canadian Council on Animal Care.

3.3.2 Tissue Collection and Preparation

All experimental animals were sacrificed via cervical dislocation, after which they were weighed for total body weight. In order to determine adiposity, fat pads (retroperitoneal and epididymal) were dissected, weighed and assessed compared to total body weight. Soleus muscle was chosen for examination in this study due to the endogenous expression of SLN. Soleus muscles were dissected, weighed, and homogenized using a handheld glass mortar and pestle homogenizer at 10:1 (vol/wt) in ice-cold PMSF buffer (250mM sucrose, 5 mM HEPES, 10mM NaN3 and 0.2 mM phenylmethanesulfonylfluoride, pH 7.5). Homogenates were frozen in liquid nitrogen and stored at -80 °C until use for future assays.

3.3.3 Western Blotting

Western Blotting was performed to determine the protein expression levels of SERCA1, SERCA2a, and SLN. Equal amounts of protein from soleus samples, along with lamelli buffer, were loaded into 7.5% glycine-based polyacrylamide gels (SERCAs) and 7.5% tricine-based polyacrylamide gels for SLN. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate proteins of interest by size. Once separated, proteins were transferred via wet (SLN) or semi-dry transfer (SERCAs) onto either a nitrocellulose (SLN) or a PVDF membrane (SERCAs). Following the transfer, the membranes were blocked in 5% skim milk or 5% BSA in TBST at room temperature for one hour. After blocking, membranes were incubated with primary antibodies overnight at 4 °C.

The membranes were then washed with TBST and incubated with the respective secondary antibody for one hour at room temperature. Membranes then underwent a final series of washes with TBST before the administration of a chemiluminescence agent. All Western blot imaging was done with a ChemiDoc Imaging System and optical density analysis was completed using Image Lab.

3.3.4 SERCA Activity

To determine SERCA function, assays determining both SERCA-mediated Ca^{2+} uptake and SERCA ATPase activity were conducted. SERCA-mediated Ca^{2+} uptake in soleus homogenates was determined by examining the rate of change in the fluorescence signals of the Ca^{2+} fluorophore, Indo-1, using a dual-emission wavelength spectrofluorometer. Indo-1 emits light at different wavelengths when bound to Ca^{2+} (485 nm) or unbound (405 nm), allowing this assay to capture the rate of Ca^{2+} uptake by analyzing the change in bound and unbound Indo-1 emissions upon the activation of SERCA with the addition of ATP (5 mM). The reaction was performed in a cuvette containing 2 mL of reaction buffer (200 mM KCl, 20 mM HEPES, 10 mM NaN3, 5 μM TPEN, and 15 mM MgCl2, pH 7.0 at 37 °C). Prior to the addition of the homogenate, background data were collected using the Felix software (Photon Technology International, Birmingham, NJ, USA). In this study, measurements of Ca^{2+} uptake rates were made without the Ca^{2+} precipitating anion, oxalate, resulting in physiologically relevant back inhibition of SERCA as Ca^{2+} accumulates inside the SR vesicle.

In the same muscle homogenates, we assessed Ca^{2+}-dependent Ca^{2+}-ATPase activity utilizing a spectrophotometric assay originally developed by Simonides and Van Hardeveld (1990) and subsequently modified for compatibility with a 96-well plate reader

(SPECTRAmax Plus; Molecular Devices, Toronto, ON)(209). In this modified protocol, reaction buffer (comprising 200 mM KCl, 20 mM HEPES at pH 7.0, 15 mM MgCl2, 1 mM EGTA, 10 mM NaN3, 5 mM ATP, and 10 mM PEP) along with 18 U/mL of both LDH and PK, was combined with homogenate and added to Eppendorf tubes containing 15 different concentrations of Ca^{2+}, ranging from 7.6 to 4.7 *p*Ca units. This study assessed ATPase activity without the presence of ionophore A23187. Similar to the oxalate-free Ca^{2+} uptake assay, omitting ionophore A23187 in this ATPase assay leads to the accumulation of Ca^{2+} within the SR vesicles. Subsequently, 100µl of each buffer/homogenate/Ca^{2+} mixture was added in duplicate to a clear-bottom 96-well plate (Costar, Corning Incorporated, NY). Finally, to remove non-SERCA related ATPase activity, 40µM of cyclopiazonic acid (CPA), a highly specific SERCA inhibitor was added to the highest [Ca^{2+}] wells, and this background activity was subtracted from the total ATPase activity measured. The reaction was initiated by adding 0.3 mM NADH, and the plate was then read at a wavelength of 340 nm for a duration of 30 minutes at 37°C. All data were then plotted against the negative logarithm of [Ca^{2+}] (*pCa*) using basic statistical software (GraphPad Prism^{TM}) to determine Vmax and EC50. EC50, the [Ca^{2+}] necessary to achieve 50% of Vmax, was determined by non-linear regression curve fitting using the sigmoidal dose response function on GraphPad. In order to accurately measure the [Ca^{2+}]s used for the ATPase activity assay, a secondary assay was performed using the Ca^{2+} fluorophore, indo-1 on a spectrofluorometric plate reader (Cytation 5 Cell Imaging Multi-Mode Reader (BioTek)) as previously described (209). The same 15 quantities of stock Ca^{2+} and all other components found in the above assay, with the exception of NADH, were added with the addition of indo-1 (1.5 µM). In addition, two other Ca^{2+} concentrations were required

for Ca^{2+} measurement, a zero Ca^{2+} and a max Ca^{2+} concentration (1mM). These were then added into a black 96 well plate (Costar, Corning Incorporated, NY) in triplicate and read on a multimodal plate reader (BioTek Cytation 5 Cell Imaging Multimode Reader) after a 15min incubation period at 37 °C. This assay is based on the difference in the maximal emission wavelength between Ca^{2+} bound indo-1 (F) and Ca^{2+} free indo-1 (G) complexes which have emission wavelengths of 405 and 485 nm upon excitation with a 355 nm wavelength, respectively. The ratio (R) of bound (F) to free (G) indo-1 complexes is used to calculate the $[Ca^{2+}]$ with the following equation:

$$[Ca^{2+}]_f = K_d * (G_{max}G_{min}) * (R-R_{min})/(R_{max}-R)$$

where K_d is 250 and represents the equilibrium constant for the interaction between Ca^{2+} and indo-1, R_{min} is the minimum value of the ratio at zero Ca^{2+}, G_{max} is the maximum value of free indo-1 at zero Ca^{2+}, R_{max} is the maximum value of the ratio at max Ca^{2+} and G_{min} is the minimum value of free indo-1 at max Ca^{2+}.

3.3.5 Comprehensive Laboratory Animal Monitoring System (CLAMS)

The Comprehensive Laboratory Animal Monitoring System (CLAMS) (Oxymax series; Columbus Instruments, Columbus, OH) is a 12 cage (20 cm x 10 cm x 12.5 cm), open circuit indirect calorimeter which allows for the sampling of air within the individual chambers. Prior to the experiment, oxygen and cardon dioxide sensors of the CLAMS were calibrated by highly purified gas. Following calibration, mice were allowed a 24-hour acclimation period in the

metabolic cages prior to data collection. Upon initiation of data collection, air samples were collected at for 30 seconds at 26-min intervals between reference readings of room air, Air samples are then used to compute the absolute and relative O_2 consumption (VO_2) and the respiratory exchange ratio (RER) of the animal over a 24-hour period. All VO_2 and RER data was reported as a 24h daily average and broken down to active and inactive periods. In addition to respiratory gas analyses, this system is equipped with X and Z activity sensors for monitoring ambulatory activity counts (when 2 adjacent X axis beams are broken in succession), rearing activity and total activity. During the study the animals had ad libitum access to water and standard mouse chow (Tekland 22/5 Rodent Diet, Harland-Tekland, Madison, WI) and food consumption was measured by weighed food before and after the experiment. The simultaneous measure of food consumption, total activity and metabolic rate were utilized to make accurate assessments of basal metabolism between WT and *Sln*$^{-/-}$ males and females. All experiments were conducted under a controlled environmental temperature of 23°C.

3.3.6 Statistical Analyses

All statistical analyses were conducted using the SPSS (version 25, IBM, Armonk, NY, USA) and graphs were generated with GraphPad Prism (version 7, GraphPad Software Inc., CA). For the comparison of SLN content, a two-tailed students T-test was conducted between male and female WT mice. In this study, it was hypothesized that the ablation of SLN would have a greater impact in females than males, as WT females were hypothesized to express greater SLN than WT males. As we expected that SLN would function similarly in both male and females but that there may be a slight dose-response phenomenon across sexes, we chose to

conduct both an ANOVA with sex and genotype as factors as well as planned comparisons to assess the impact of SLN ablation in each sex. The planned comparisons consisted of a Bonferroni test between genotypes (WT and $Sln^{-/-}$) for each sex. Similarly, when a significant interaction effect was found in the ANOVA, a Bonferroni test was conducted to assess differences across sex and genotype groups. The significance level was set at 0.05 for all tests and all values are represented as a mean ± SEM.

3.4 Results

3.4.1 Expression of SERCAs and SLN

Western blotting was conducted on the soleus muscle homogenates from male and female, 4–6-month-old WT and *Sln*$^{-/-}$ mice in order to discern if sex impacted the expression of SLN and SERCAs. SLN content was 2.7-fold greater in the soleus muscles of WT female animals compared to males (Fig. 4A, 1.57 ± 0.29 vs. 0.58 ± 0.10, p = 0.004). When examining the content of SERCA1a (Fig. 4B), neither sex (p = 0.85) nor SLN expression (p = 0.70) were found to be significant factors and there were no significant differences between pairwise comparisons. Conversely, there was a significant interaction between sex and SLN ablation in the expression of SERCA2a (Fig. 4C, p = 0.04), wherein females displayed greater SERCA2a content than males in WT animals (0.007 ± 0.001 vs. 0.002 ± 0.001, p = 0.02), with no differences between *Sln*$^{-/-}$ groups (0.003 ± 0.001 vs 0.002 ± 0.001, p = 0.99). Pairwise comparisons also revealed that WT females displayed significantly greater SERCA2a content than *Sln*$^{-/-}$ females (0.007 ± 0.001 vs. 0.003 ± 0.001, p = 0.04), with no differences between WT males and *Sln*$^{-/-}$males (0.002 ± 0.001 vs. 0.002 ± 0.001, p = 0.80).

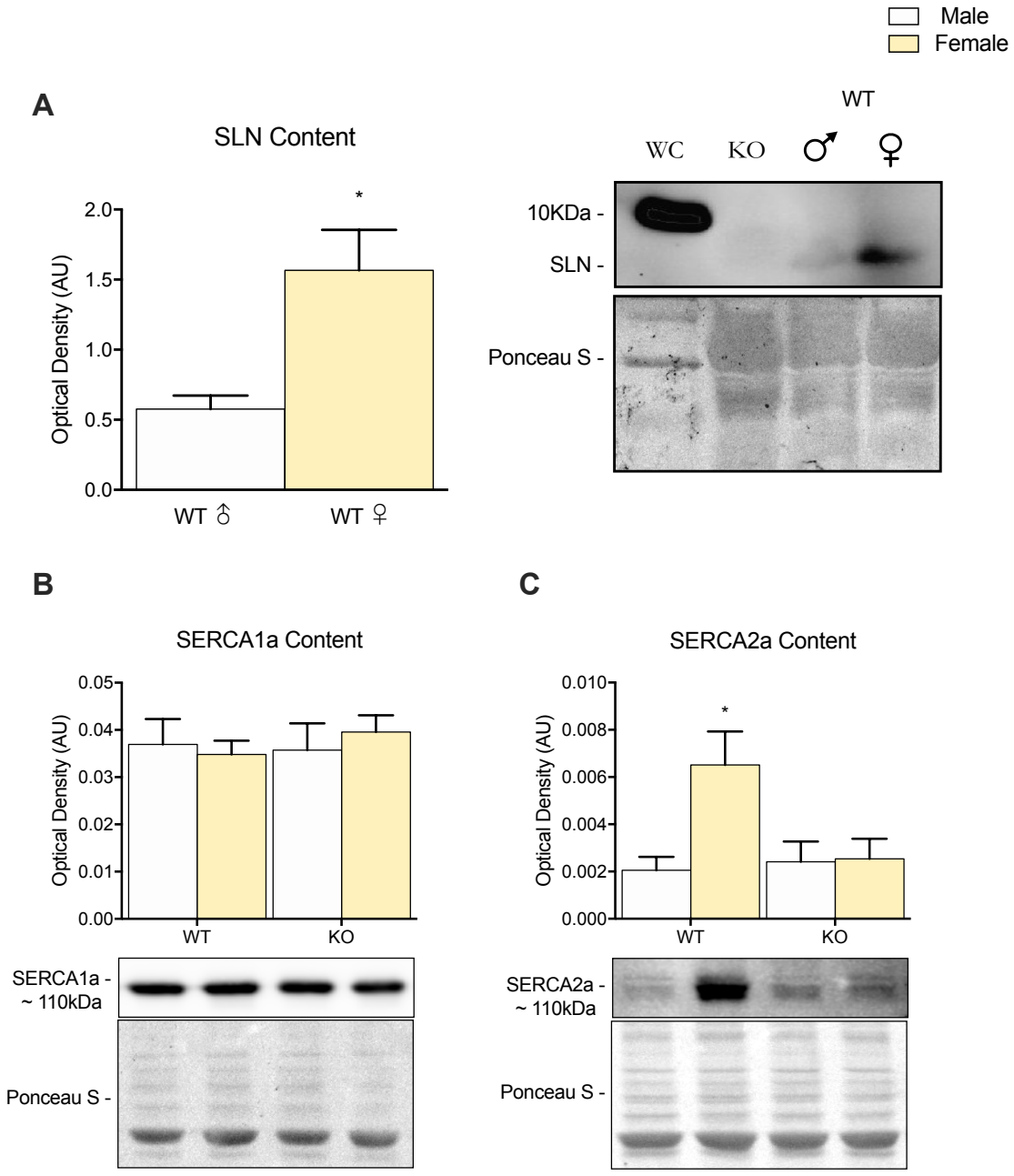


Figure 4. Sex differences in SLN and SERCA content in young adult healthy mice.

Sex differences in SLN content was determined by Western blotting soleus muscle homogenates of healthy young adult (4-6M) male and female wild type (WT) mice (A). The representative SLN blot shows the 10KDa molecular weight marker (Western C, WC), a *Sln*$^{-/-}$ (KO) sample and male WT and female WT sample. Western blotting was also conducted to examine the content of SERCA1a (B) and SERCA2a (C) in healthy young adult male and female WT and KO animals. Optical density for each band of interest was normalized to total protein using Ponceau S staining. * Denotes the group is significantly different from all other groups, $p \leqq 0.05$. Data are mean ± SEM (n=6).

3.4.2 SERCA Activity

To investigate the effect of sex and SLN expression on SERCA function, we assessed SERCA-mediated Ca^{2+} uptake and SERCA ATPase activity, as well as the apparent coupling ratio of SERCA in male and female, WT and *Sln*$^{-/-}$ mice. This study assessed SERCA Ca^{2+} uptake in the absence of the Ca^{2+} precipitating anion, oxalate, leading to the back-inhibition of SERCA pumps as Ca^{2+} accumulated within SR vesicles, an environment that is physiological relevant to that of skeletal muscle (Fig. 5A). In the present study, neither sex ($p = 0.284$) nor SLN ablation ($p = 0.175$) impacted Ca^{2+} uptake at *p*Ca 6.0 (Fig. 5B). Conversely, while sex was not a significant factor (Fig. 5C, $p = 0.290$), SLN expression had a significant effect on SERCA Ca^{2+} uptake at *p*Ca 5.7 ($p = 0.049$), with *Sln*$^{-/-}$ animals generally displaying greater Ca^{2+} uptake than males. Here, pairwise comparisons revealed that female *Sln*$^{-/-}$ mice displayed greater Ca^{2+} uptake than WT females (5.991 ± 1.36 vs. 3.225 ± 0.909, $p = 0.050$) which did not reach significance between male groups (4.177 ± 0.890 vs. 2.996 ± 0.274, $p = 0.385$).

Similar to uptake, Ca^{2+}-activated SERCA ATPase activity was measured in the presence of a Ca^{2+} gradient (without the use of ionophore A23187) in the same soleus muscle homogenates at a *p*Ca of 5.7 and 6.0 (Fig. 6A). Here, it was found that neither sex nor SLN ablation were significant factors in altering SERCA ATPase activity at *p*Ca 6.0 (Fig. 6B, $p = 0.314$ & $p = 0.611$, respectively). Similar findings were found at *p*Ca 5.7, as sex and genotype of the animals did not statistically impact ATPase activity (Fig. 6C, $p = 0.502$ & $p = 0.902$, respectively).

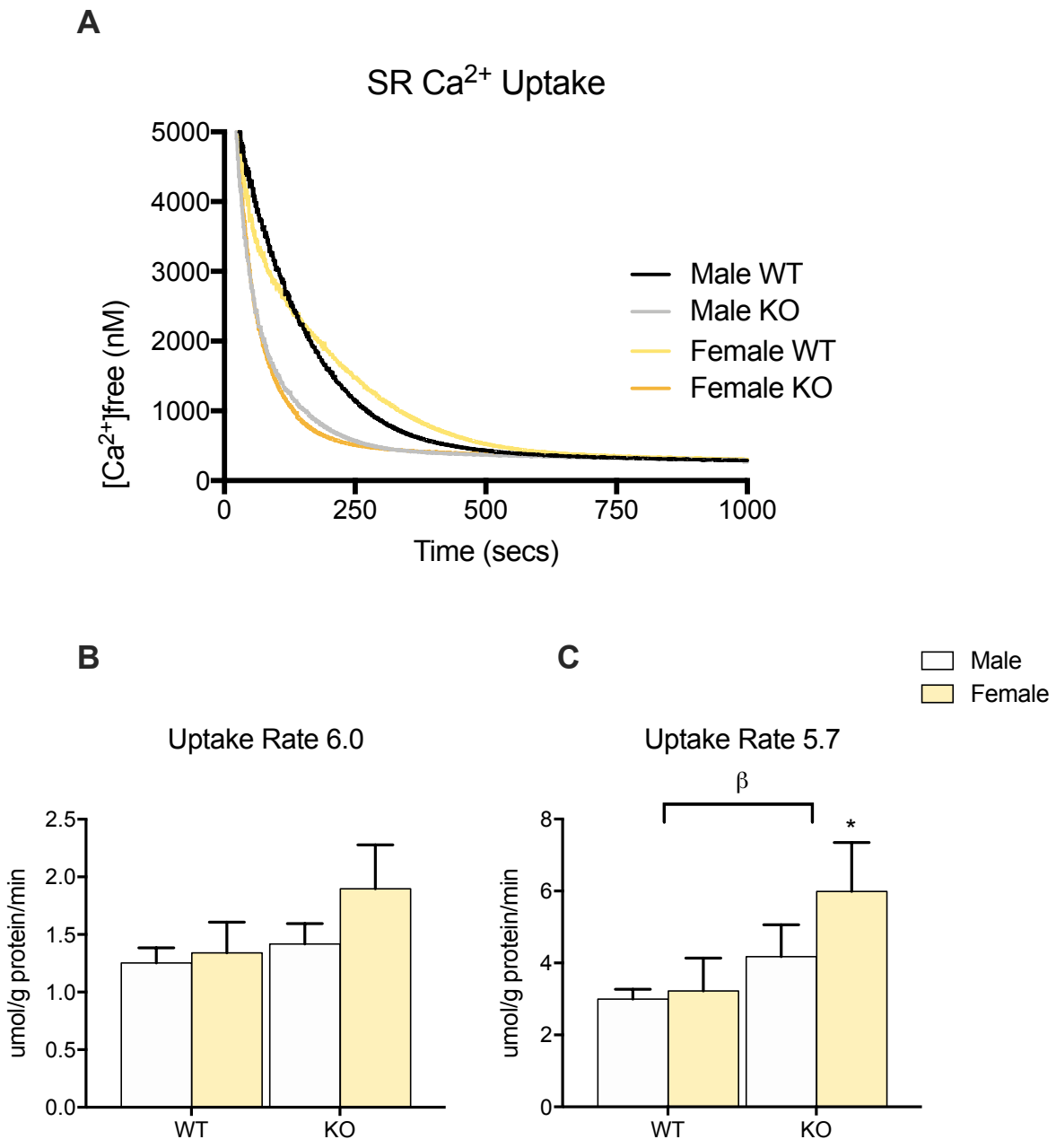


Figure 5. The impact of SLN on SERCA-mediated Ca^{2+} Uptake between sexes.

SERCA-mediated Ca^{2+} uptake was assessed in soleus muscle homogenates isolated from young adult (4-6M), male and female, wild type (WT) and *Sln*$^{-/-}$ (KO) mice in the presence of a Ca^{2+} gradient (A). The rate of Ca^{2+} uptake expressed in µmol•g protein•min was measured by capturing the slope at *p*Ca 6.0 equating to 1000µmol (B) and *p*Ca 5.7 equating to 2000 µmol. ß Denotes a main effect of genotype, * significantly different than WT group of same sex, $p \leq 0.05$. Data are mean ± SEM (n=6).

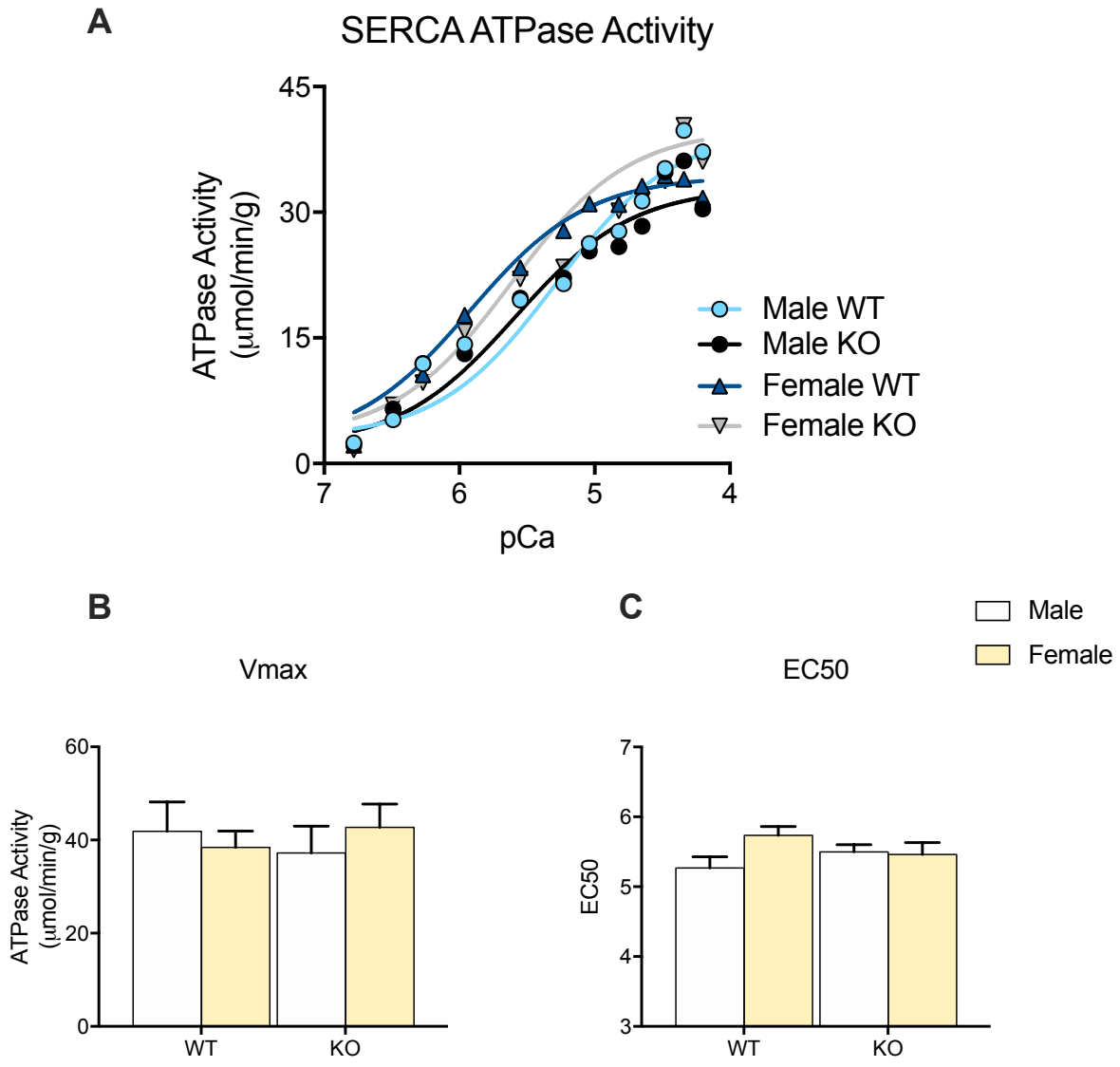


Figure 6. The impact of SLN on SERCA ATPase activity between sexes.

Ca^{2+}-ATPase activity was analyzed over Ca^{2+} concentrations ranging from *p*Ca 6.78 to *p*Ca 4.20 in soleus muscle homogenates of wild type (WT) and *Sln*$^{-/-}$ (KO), male and female mice. Average ATPase activity curves are displayed against a negative logarithm of Ca^{2+} concentrations (A). Vmax is the maximal SR Ca^{2+}ATPase activity expressed as µmol•g protein•min (B). EC50 is the negative log of the Ca^{2+} concentration required to attain the half-maximal Ca^{2+}-ATPase activity rate (C). $p \leq 0.05$. Data are mean ± SEM (n=6).

The apparent coupling ratio is a measure of the Ca^{2+} transport efficiency of SERCA and is calculated by dividing the Ca^{2+} uptake rate by the ATPase activity at a specific molar [Ca^{2+}] (*p*Ca 5.7 and 6.0). As mentioned above, at the higher [Ca^{2+}] of *p*Ca 5.7, SLN ablation resulted in greater Ca^{2+} uptake with no change to ATPase activity (Fig. 7A & C). This resulted in $Sln^{-/-}$ animals having a higher coupling ratio compared with WT animals at *p*Ca 5.7 (Fig. 7E, p = 0.030). Pairwise comparisons found $Sln^{-/-}$ females displayed a significantly greater coupling ratio than WT females (0.264 ± 0.05 vs. 0.131 ± 0.02, p = 0.05), a phenomenon which did not reach significance in the male groups (0.255 ± 0.07 vs. 0.176 ± 0.03, p = 0.23). At *p*Ca 6.0, there were no differences in Ca^{2+} uptake or ATPase activity due to sex or SLN ablation (Fig. 7B & D). However, like *p*Ca 5.7, SLN ablation significantly increased the coupling ratio at *p*Ca 6.0 (Fig. 7F, p = 0.033). Again, pairwise comparisons revealed that female $Sln^{-/-}$ muscle displayed greater coupling ratios than female WT, (0.129 ± 0.02 vs. 0.079 ± 0.01, p = 0.05), while males did not reach significance (0.125 ± 0.02 vs. 0.094 ± 0.01, p = 0.28).

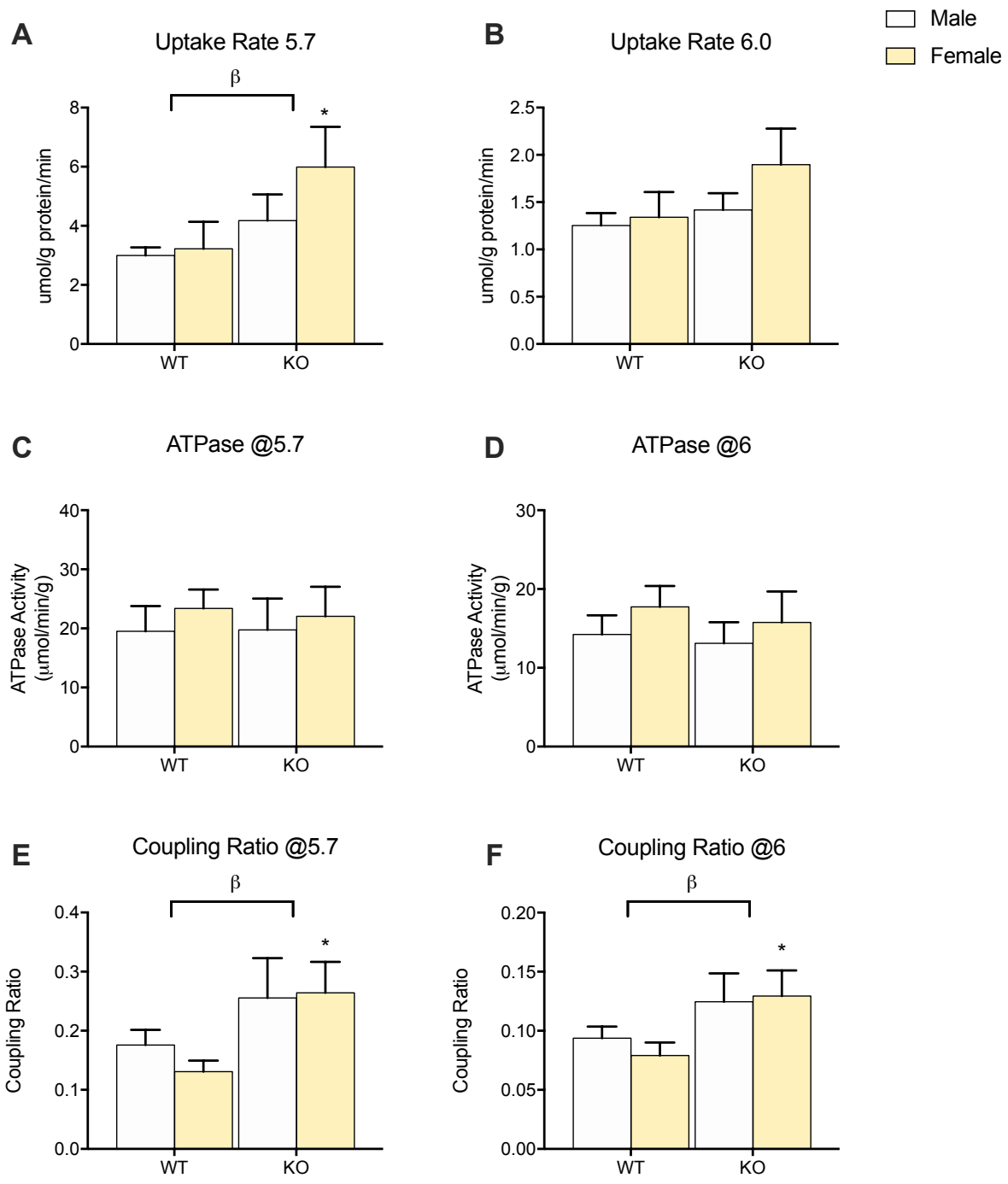


Figure 7. SLN and the SERCA coupling ratio between male and female mice.

Average values for Ca^{2+} uptake without oxalate (A & B), Ca^{2+} ATPase activity without Ionophore A23187 (C & D) and apparent coupling ratio (Ca^{2+} uptake / Ca^{2+}-ATPase activity, E & F) in the soleus muscle homogenates of male and female, WT and $Sln^{-/-}$ (KO) mice. ß denotes main effect of genotype. * significantly different than WT group of same sex. $p \leqq 0.05$. Data are mean ± SEM (n=6).

3.4.3 Whole Body Energy Expenditure, Activity and Food Intake

To determine the effect of SLN on whole body metabolic expenditure in both sexes, adult male and female, WT and *Sln*$^{-/-}$ mice were put into metabolic cages, referred to as the CLAMS (Comprehensive Laboratory Animal Monitoring System), which collected respiratory gases and measures of activity levels (Table 1). Before and after the CLAMS protocol, animals were weighed for whole body weight and adiposity was determined at the time of dissection. When examining animal body weights, sex was a significant factor ($p < 0.001$), with females displaying lower body weights than males. Genotype did not have a significant impact on body weight ($p = 0.57$). Conversely, genotype was a statistically significant factor for adiposity ($p = 0.03$), while sex did not reach statistical significance ($p = 0.11$). Pairwise comparisons revealed that *Sln*$^{-/-}$ females displayed greater adiposity than WT females (3.97 ± 0.85 vs. 2.36 ± 0.16, $p = 0.03$), whereas the adiposity of male *Sln*$^{-/-}$ and WT animals was not different (2.69 ± 0.41 vs. 1.97 ± 0.34, $p = 0.32$). In order to understand habitual food consumption, food intake was measured during the CLAMS protocol. Again, sex significantly impacted food intake ($p < 0.001$), with females displaying lower food intake than males. Converse to sex, SLN ablation did not reach significance for food intake ($p = 0.08$).

After acclimation in the CLAMS cages, data was captured for 24hr (12hr light:12hr dark) and all data are represented as a daily average and further broken down into the average of active and inactive periods (Table 1). For measures of oxygen consumption (VO_2), data are expressed as both an absolute value (mL O_2/hr) and relative to body weight (mL O_2/kg/hr). In the present study, main effects of both sex and genotype for all VO_2 measures were found ($p < 0.01$ for each). For each measure of absolute VO_2 (daily, active and inactive), pairwise

comparisons found that SLN ablation resulted in lower absolute VO_2 compared to WT animals of the same sex ($p < 0.01$ for all WT vs. $Sln^{-/-}$, male and female pairs). Similarly, sex was a significant factor in absolute VO_2 ($p < 0.01$), where females generally displayed lower absolute VO_2 than male counterparts. However, in two measures of absolute VO_2, the inactive and total daily averages, there was a sex*genotype interaction ($p = 0.03$ and $p = 0.04$, respectfully). Post hoc comparisons revealed WT females displayed statistically lower absolute VO_2 compared to their WT males ($p < 0.01$ for both), whereas there were no differences between $Sln^{-/-}$ females and males (daily $p = 0.32$, inactive $p = 0.07$). When VO_2 was normalized to body weight, there were significant main effects of sex and genotype (main effect $p < 0.01$ for all), with no significant interaction effects. Converse to absolute VO_2, females generally displayed greater relative VO_2 than males. With respect to the effect of SLN ablation on relative VO_2, pairwise comparisons revealed that $Sln^{-/-}$ animals displayed lower VO_2 than WT animals for daily, active, and inactive periods ($p < 0.001$ for all WT vs. $Sln^{-/-}$, male and female pairs).

Respiratory exchange ratio (RER), a ratio of CO_2 production to O_2 consumption, was assessed as an indicator of fuel utilization and displayed as a total daily average and an average of active and inactive periods. Sex was a significant factor in daily RER ($p = 0.001$) and inactive RER ($p <0.001$) but was not statistically significant when animals were active ($p = 0.084$). In the daily average and inactive periods, females generally displayed lower RER than males, regardless of genotype. As changes in activity levels directly impact VO_2 and RER, activity levels were measured using dual beam lasers, where any single beam break was attributed to total activity and dual beam breaks to ambulatory movement. Here, it was found that neither sex nor SLN ablation affected total activity ($p = 0.27$ and $p = 0.40$). Similarly, no

differences were found in the ambulatory activity between sexes or with SLN ablation ($p = 0.25$ and $p = 0.78$, respectively).

Table 1. Adaptations to basal metabolism with SLN ablation between sexes.

		WT Male	*Sln*$^{-/-}$ Male	WT Female	*Sln*$^{-/-}$ Female
Body Mass (g)	#	32.98 ± 0.48	31.51 ± 0.32	22.52 ± 0.16	23.16 ± 0.43
Adiposity %	ß	1.97 ± 0.34	2.69 ± 0.41	2.36 ± 0.16	3.97 ± 0.85 ^
Food Intake (g)	#	5.45 ± 0.31	4.76 ± 0.34	4.07 ± 0.39	3.49 ± 0.35
Absolute Energy Expenditure (mL O_2/hr) # ß for all					
Daily VO_2	X	124.83 ± 2.66	85.53 ± 2.06 *	97.11 ± 6.18 *	76.20 ± 3.28 ^
Active VO_2		139.86 ± 2.99	97.29 ± 2.49 *	113.57 ± 2.65	87.14 ± 2.93 ^
Inactive VO_2	X	112.35 ± 2.59	76.47 ± 1.74 *	85.41 ± 2.20 *	66.41 ± 2.70 ^
Relative Energy Expenditure (mL O_2/kg/hr) # ß					
Daily VO_2		3798.0 ± 95.6	2716.1 ± 64.8 *	4304.4 ± 180.2	3318.1 ± 153.1 ^
Active VO_2		4259.6 ± 129.5	3063.7 ± 78.3 *	5035.1 ± 219.0	3786.6 ± 148.4 ^
Inactive VO_2		3414.7 ± 79.8	2412.1 ± 58.2 *	3784.6 ± 151.8	2883.1 ± 138.2 ^
Respiratory Exchange Ratio (RER)					
Daily RER	#	0.948 ± 0.012	0.931 ± 0.013	0.886 ± 0.014	0.893 ± 0.014
Active RER		0.959 ± 0.010	0.932 ± 0.015	0.911 ± 0.016	0.924 ± 0.018
Inactive RER	#	0.941 ± 0.013	0.931 ± 0.013	0.882 ± 0.013	0.889 ± 0.015
Activity					
Total Activity		5968.3 ± 839.4	7431.6 ± 428.1	7662.7 ± 1129.8	7627.4 ± 585.1
Ambulatory		3130.4 ± 929.1	2288.1 ± 201.8	3682.7 ± 900.0	3983.6 ± 795.1

Average CLAMS data collected from a 24hr cycle (12hr light:12hr dark). Active periods were discerned using activity data, with a cut off of >15 beam breaks per 30 second sample collection. # Denotes main effect of sex and ß main effect of genotype and X denotes a sex by genotype interaction. * Denotes a significant difference from WT males, ^ Denotes a significant difference from WT females, $p \leqq 0.05$. Data are mean ± SEM (n=7).

3.5 Discussion

Previous research examining SLN in animal models has exclusively reported on males and this study sought to address this sex bias by investigating SLN expression and function in both males and females. In the present study, it was hypothesized that SLN expression would be greater in females as previous research has linked SLN expression with delayed relaxation rates, improved fatigability and greater lipid oxidation, all traits which are characteristic of female muscle (168-177, 197-199). In alignment with this hypothesis, greater SLN expression was found in the soleus muscles of female WT mice compared to their male WT counterparts. Importantly, greater SLN expression in females was observed without changes to the content of SERCA1a, suggesting a greater SLN:SERCA ratio and increased SERCA inhibition in females.

To determine how SLN expression impacts SERCA function in both sexes, SERCA ATPase activity and SERCA Ca^{2+} uptake were assessed in the soleus muscles of male and female, WT and *Sln*$^{-/-}$ mice. Both ATPase and Ca^{2+} uptake rates were examined under conditions allowing for back inhibition of SERCA which is more indicative of *in vivo* SERCA function. In line with previous research (45, 57), SLN was shown to uncouple SERCA-mediated Ca^{2+} uptake from ATPase activity in the present study, as *Sln*$^{-/-}$ animals displayed lower Ca^{2+} uptake with similar ATPase activity compared to WT animals. While there were no statistically significant differences between male and female groups, the ablation of SLN seemed to impact SERCA function to a greater extent in females than males. Specifically, compared with WT, the coupling ratio at a *p*Ca of 5.7 was higher in *Sln*$^{-/-}$ mice by 2.01-fold in females and only 1.45-fold in males. Similar findings were observed at lower [Ca^{2+}] (*p*Ca 6.0),

with a 1.63-fold and 1.33-fold greater coupling ratio in *Sln*$^{-/-}$ compared with WT mice, in females and males, respectively. Greater disparities in SERCA function between *Sln*$^{-/-}$ and WT females along with greater SLN expression in females collectively suggests that there is greater endogenous inhibition and uncoupling of SERCA by SLN in the soleus muscle of females compared with males.

As both previous literature (45, 46, 48) and the present study have shown SLN reduces SERCA efficiency, this study also sought to determine how whole-body energy expenditure was impacted with SLN ablation in both males and female mice. To assess whole body energy expenditure, indirect calorimetry was conducted by placing mice in metabolic cages known as the Comprehensive Laboratory Animal Monitoring System (CLAMS). The CLAMS periodically samples cage O_2 and CO_2 exchanges to determine VO_2 and RER in conjunction with the measurement of habitual activity, and food intake measures. Body weight and body composition was measured by examining total body weight and adiposity (fat pad mass: total body mass). Several findings from these analyses align with established sex differences in the body composition and metabolism literature. Specifically, female mice displayed greater adiposity and lower body mass, food intake, and RER than males, aligning with previous work with both humans and animals (210-212).

The literature examining sex differences in absolute VO_2 is congruent in both humans and animals, with greater absolute VO_2 in males attributed to their greater body and fat free mass. In this study, absolute VO_2 was separated across active and inactive periods and reported as active, inactive, and daily (i.e. active and inactive combined over 24hrs). Absolute VO_2 was lower in female WT mice compared to their male counterparts during both active and inactive

periods and, as a result, the 24hr daily VO_2 was also lower in females. However, the pattern of sex differences was different in *Sln*$^{-/-}$ mice, where a significant interaction was observed between sex and genotype for two measures of absolute VO_2, namely the inactive and daily averages. These novel findings could suggest that sex differences in absolute VO_2 are predicated on the expression of SLN, however it is likely more reflective of discrepancies in lean body mass between sexes. As SLN uncouples SERCA function in skeletal muscle and males display greater muscle mass than females, it is logical that the ablation of SLN would have a greater impact on absolute VO_2 in males than females. Accordingly, the average daily absolute VO_2 of *Sln*$^{-/-}$ males was only 65.4% of WT males, whereas in *Sln*$^{-/-}$ females it was 79.1% of WT females, effectively eliminating the statistical sex differences in absolute VO_2 between male and female *Sln*$^{-/-}$ animals. When VO_2 was normalized to body weight the sex*genotype interactions were eliminated, which aligns with the notion that greater muscle mass amplifies the impact of SLN on absolute whole-body energy expenditure. It should be noted that sex remained a significant factor with females generally displaying greater relative VO_2 than their male counterparts, a commonly reported finding across various mouse strains (212). Notably, none of the sex differences in relative and absolute VO_2 can be explained by differences in activity levels, as both total and ambulatory activities were similar between male and female mice of the same genotype.

The present study also reported a significant effect of SLN ablation on energy expenditure. In fact, genotype was a significant factor for all measures of both absolute and relative VO_2, with statistical significance found between *Sln*$^{-/-}$ and WT animals in both males and females for each of the daily, active and inactive comparisons. Interestingly, the data from

this study suggest that SLN ablation has a greater impact on measures of VO_2 than previously reported. Previous research by Bombardier et al (2013) examining whole-body VO_2 between male WT and *Sln*$^{-/-}$ mice found no differences in relative VO_2 with SLN ablation when mice were fed a standard chow diet (48). The discrepancy in relative VO_2 in male mice between studies is especially interesting given the work by Bombardier et al (2013) was conducted by previous members of the Tupling lab using the same animal line and CLAMS system. A potential explanation of the variation in study findings is the environment in which the experiments were run. At the University of Waterloo, murine lines are maintained in the Central Animal Facility (CAF) with a photic cycle set to light from 7am to 7pm (resting phase) and dark from 7pm to 7am (active phase). During the time of data collection for the Bombardier et al (2013) study, the CLAMS system was located in Burt Matthews Hall (BMH) which required the relocation of animals across campus and acclimation to the opposite 12hr:12hr light dark cycle (8am to 8pm dark/active, 8pm to 8AM light/resting). Subsequently the CLAMS system was relocated to the CAF where the data was collected for this study. While extensive acclimation was done in both studies and data was collected over full 24hr, light and dark cycles, it is possible that the variation in environment and the stress of relocation may alter animal behaviour and energy expenditure. Notably, the relative daily VO_2 data reported by Bombardier et al (2013) was higher in *Sln*$^{-/-}$ males than that of this study (2965 ± 52 vs 2412.1 ± 58), which may be attributed to greater ambulatory activity (2723 ± 146 vs. 2288 ± 201). Variation in ambulatory activity in laboratory animals is common and the findings here may be attributable to the transition in photic cycles (213), transportation stress (214) or to circannual oscillations in activity that has been previously reported in laboratory C57BL/6 mice (215). However, if this environmental stress was the cause of the disparities between

studies it would be assumed that differences in ambulation would also be witnessed in WT males, as both genotypes underwent the same protocol. Contrary to *Sln*$^{-/-}$ males, the relative daily VO_2 of WT males in Bombardier et al (2013) was lower than that of this study (3000 ± 71 vs 3414.7 ± 79.8). The etiology of the differences in WT males between studies is harder to discern as it was not accompanied by differences in activity level (3262 ± 302 vs. 3130 ± 929) (48). While speculatory, genetic drift through regular breeding may have occurred over the 15+ years between studies which could account for the disparities in energy expenditure findings. Specifically, greater SLN expression in WT mice in the present study would account for higher relative VO_2 in the absence of changes to activity levels. Similarly, SLN could have been reduced in the study by Bombardier et al (2013) due to the shift in light:dark cycle or transportation stress. Previous research has reported that SLN expression is upregulated in response to excess caloric intake (52, 57) and while research has yet to examine how caloric restriction impacts SLN expression, reduced feeding in response to circadian rhythm manipulation and stress could theoretically result in reduced SLN expression. Without directly measuring SLN expression between study samples it is challenging to discern if this hypothesis is true, however future studies should examine how caloric restriction impacts SLN expression and should take care to extensively report on protocol procedures, environmental factors and animal husbandry.

Given the previously published data by Bombardier et al (2013)(48), the present study had hypothesized that SLN ablation would not impact whole body energy expenditure in male mice. In contrast, this was the first study to assess energy expenditure between WT and *Sln*$^{-/-}$ female mice and as it was hypothesized WT females would express greater SLN content than

male counterparts, it was also anticipated that SLN ablation would have a greater impact on whole body energy expenditure between female groups. In the present study, SLN expression significantly impacted energy expenditure in both sexes, with significantly lower VO_2 for *Sln*$^{-/-}$ mice compared to WT in all measures of absolute and relative VO_2 without changes to activity levels. While the VO_2 data from males in the present study was in contrast to the hypothesis and the previously reported data, the VO_2 data from females may be reflective of other models which display greater SLN expression. Another study by Bombardier et al (2013) reported a 3-fold increase in SLN expression in the soleus muscle of WT males in response to an 8-week high fat (HF) diet (57). In contrast to chow fed males, Bombardier et al (2013) reported lower relative VO_2 in HF-fed male *Sln*$^{-/-}$ mice than HF-fed WT males, which was accompanied by significantly greater adiposity in the HF-fed *Sln*$^{-/-}$ males (57). Interestingly, the present study presented similar adiposity findings, with SLN expression significantly impacting adiposity, despite similar food intake and overall body weight between genotypes. Specifically, female *Sln*$^{-/-}$ animals displayed significantly greater adiposity than WT females, which did not reach significance between male groups. This aligns with the data from Bombardier et al (2013) wherein chow fed males displayed no differences in adiposity, while discrepancies in adiposity were only witnessed following HF feeding and increased SLN expression (57). Collectively the reports on chow and HF fed males and along with the data on females from the present study suggest that as the expression of SLN increases so does its impact on SERCA function and whole-body energy expenditure. This is especially intriguing as humans and larger mammals are reported to display over 10-fold greater SLN expression than rodents (27) which could indicate the impact of SLN expression in humans is even greater than the present study suggests. Further examination of SLN as a therapeutic target for

increasing energy expenditure in humans may have wide-ranging implications as greater adiposity is an independent risk factor for cardiovascular disease, type II diabetes, cancer, sleep apnea, arthritis and all-cause mortality (216, 217).

In conclusion, this study aimed to determine if SLN content differed between males and females and sought to examine the impact of SLN ablation on SERCA efficiency and whole-body energy expenditure between sexes. This study presented the novel findings that SLN content was greater in the soleus muscle of female mice compared to males. In accordance with the literature, this study also found SLN ablation increased the coupling ratio of SERCA. This was especially evident in females, which aligned with the greater SLN expression. The ablation of SLN also impacted whole-body energy expenditure and adiposity in this study. While all measures of whole-body VO_2 were reduced with SLN ablation, the current study found that male *Sln*$^{-/-}$ mice displayed a greater reduction in absolute VO_2 compared to WT males than that witnessed between female groups. This was attributed to greater muscle mass as this phenomenon was not present when VO_2 was normalized to body weight. Finally, SLN ablation was associated with adiposity in females, despite similar habitual food intake. Together the results of this study confirm the role of SLN in reducing SERCA efficiency and reported the novel findings that females display greater SLN expression. As such, this study makes a potent argument for the inclusion of females in future studies examining the role of SLN in SERCA function and energy expenditure.

Chapter 4: The Role of SLN in SERCA Protection with Aging in Male and Female Mice

4.1 Abstract

Oxidative stress, resulting from an imbalance between the production of reactive oxygen and nitrogen species (RONS) and antioxidant defense systems, is a prominent feature of aging and has been linked to age-related muscle dysfunction. Several inherent features of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pump make it vulnerable to oxidative damage and age-related oxidative stress has been shown to target SERCA, leading to altered Ca^{2+} transport and homeostasis. Recent evidence has shown that sarcolipin (SLN), a small protein inhibitor of SERCA, can protect the pump from damage by RONS and that SLN is dynamically upregulated in models of muscle unloading and disease. Furthermore, previous work by our lab has demonstrated that females, who display inherent resistance to oxidative damage, display greater levels of SLN expression than male counterparts. In order to examine if SLN is similarly recruited to protect SERCA with aging and if the sexual dimorphism in SLN content persists with age, this study examined the content of SLN, SERCA1a and SERCA2a in the soleus muscles of male and female WT mice at two age groups: adult (4-6 months) and older adult (18+ months). To test the hypotheses that SLN offers protection against SERCA oxidative damage with aging and that aged WT females will display greater SERCA protection due to greater SLN expression than aged WT males, this study assessed SERCA function (ATPase activity and SERCA mediated Ca^{2+} uptake) and SERCA damage between adult and older adult, male and female, WT and $Sln^{-/-}$ animals. Aging reduced SERCA1a content in both males and females and increased SERCA2a content in males only.

While sex had no significant impact on SERCA function, females generally displayed greater SERCA1a damage than males. As expected, aging resulted in reduced SERCA function and increased SERCA damage in all sex and genotype groups. SLN content was significantly greater in females regardless of age and in older animals compared to younger counterparts regardless of sex. SLN ablation improved SERCA Ca^{2+} affinity regardless of age and had no significant impact on SERCA damage, suggesting that SLN does not play a role in protecting SERCA from age- induced oxidative damage. This study is the first to begin elucidating a role for the upregulation of SLN with aging and while SLN had little impact on SERCA protection with aging, future research should seek to examine if SLN is dynamically upregulated in aging in order to promote energy expenditure and/or the maintenance of muscle mass to combat sarcopenic obesity.

4.2 Introduction

Oxidative stress, resulting from an imbalance between RONS production and antioxidant defense systems, is a hallmark of aging and has been implicated as a key contributor to age-related muscle dysfunction (116, 122, 123). Excessive RONS production with aging is thought to occur mainly due to mitochondrial dysfunction (218). Increased electron leakage from the electron transport chain results in the generation of superoxide anion ($O_2 \cdot$-) within the mitochondria, which can further react with nitric oxide (NO) to form peroxynitrite (ONOO-), a potent RNS. In addition to mitochondrial dysfunction, RONS generation may be exacerbated by the infiltration of immune cells and subsequent activation of inflammatory signalling (219-221), as well as Ca^{2+} dysregulation which activates RONS producing enzymes such as nitric oxide synthase (NOS) (153). With respect to antioxidant activity, most studies show an increase in the activity of several antioxidant enzymes (SOD, CAT, GPx) with aging (222, 223), albeit this may be fibre type specific with oxidative muscles showing a greater antioxidant response with aging than glycolytic muscles (224, 225). While antioxidant activity may be elevated with aging, it is clear that elevations in RONS production outweigh the antioxidant capacity as oxidative damage to proteins, lipids and DNA is commonly witnessed in aged skeletal muscle (115, 153, 201, 226, 227).

A growing body of evidence suggests that females may be protected from cellular damage due to oxidative stress compared to their male counterparts. Borras et al (2003) reported that ROS production was greater in mitochondria isolated from male rats, while mitochondria from female rats displayed higher levels of the antioxidant, glutathione (GSH) (228). Interestingly, in this same study mitochondria from ovariectomized female rats

displayed increased ROS and decreased levels of GSH, similar to that seen in male rats, which was improved when treated with exogenous estrogen (228). Later studies have shown that 17β-estradiol suppresses oxidative stress in the face of cell injury by promoting the expression of antioxidant genes, including heme oxygenase-1(HO-1), nuclear factor erythroid-2 (Nrf2), and sirtuin 1 (SIRT1) (229-231). While it is clear that estrogen plays a role in protection against oxidative stress, the sex differences in resistance to oxidative stress persists in postmenopausal female animals and humans. A study by Pansarasa et al (2000) found that oxidized glutathione (GSSG), a marker of oxidative stress, was significantly greater in the skeletal muscle biopsies of older adults (66-91 years old) than young adults (17-40 years old) and that older adult males displayed higher levels than older adult females (200). Correspondingly, aged male rats display lower mitochondrial respiratory chain function, accompanied by a similar level of ROS production and lower antioxidant enzyme activities than female counterparts (202). While research is still uncovering the mechanisms underlying the resistance to oxidative stress in aging females, lower levels of ROS and greater antioxidant capacity has been associated with longevity in drosophila, rodents and multiple mammalian species, including humans, which aligns with longer lifespans witnessed in females than males (203, 232, 233).

Age-related oxidative stress has been shown to target the SR and SERCA, resulting in changes to Ca^{2+} transport and homeostasis. Lipids within the SR bilayer have been shown to be more susceptible to peroxidation with aging, resulting in increased Ca^{2+} permeability of the SR membrane (226). In addition to peroxidation of SR membrane lipids, the lipid composition and fluidity of the SR has been shown to be altered with aging (226, 234), and previous evidence suggests that these changes can directly impact the SR Ca^{2+} transport by limiting the

rotational movement of SERCA (235). In support of this notion, a study by Fano et al (2001) found increases to oxidation of lipids in the vastus lateralis of older adults compared to younger adults, which was accompanied by reductions in RYR function in aged males and females and reductions in SERCA activity, specifically in aged males (201). Furthermore, the proximity of SERCA to reactive lipid species may also result in oxidative damage to the pump itself. Indeed, 4-hydroxynonenal (4HNE), a product of lipid hyperoxides, has been shown to directly impair SERCA function even in the absence of other SERCA oxidative damage (236).

In addition to its location within the SR membrane, SERCA is vulnerable to oxidative damage due to its inherent function and structure. While the majority of RONS may be generated by mitochondrial dysfunction with aging, the metabolic nature of ATPase activity can result in the generation of $O_2 \cdot -$ by xanthine oxidase, as downstream byproducts of ATP hydrolysis (237, 238). Given the close association between SERCA's enzymatic activity and RONS production, it may increase the likelihood of oxidative damage to SERCA itself. Moreover, the structure of SERCA also makes it liable to oxidative and nitrosative damage. As mentioned previously, SERCA contains multiple highly reactive Cys and Tyr residues that are susceptible to redox modifications which directly affect SERCA's structure and function (116). Tyr294 and Tyr295, which lie within the M4 transmembrane region, undergo nitrosative damage with aging, which has been associated with reductions in SERCA function (118, 158). Similarly, mass spectrometry analysis has also revealed age-dependent oxidative modification of cysteine residues located at key functional sites, attributing to the loss in SERCA function witnessed with aging (239). Together these data provide evidence that SERCA is a target for age-related oxidative and nitrosative damage, and thus methods to protect SERCA function in

the face of oxidative stress may prove fruitful in improving SR Ca^{2+} uptake and SR Ca^{2+} load, ultimately aiding in restoring both Ca^{2+} homeostasis and muscle function.

Studies examining the use of antioxidant treatment to protect SERCA function in aged animals have yielded mixed results. Smith et al (2015) examined the effects of treatment with the antioxidant, buthionine sulfoximine, on diaphragm function in adult and aged rats, finding improvements to SERCA activity and muscle function with antioxidant treatment in adult muscle but irreversible damage and reduced to SERCA function with advanced aging(127). Conversely, upregulation of mitochondrial antioxidant activity via genetic overexpression of the human catalase gene within the mitochondria of aged mice has proven beneficial in restoring SR Ca^{2+} load and improving muscle function compared to age matched WT animals (240). Given that the structural integrity of SERCA is essential for its proper function, small chaperone proteins have also been examined for their role in stabilizing and protecting SERCA from oxidative damage. Recent studies have shown that HSP70, PLN and SLN are able to prevent thermal inactivation of SERCA in HEK-293 cells when heat stressed (130-132). Specifically, Fu et al (2020) found that both SLN and PLN have capacity to protect SERCA2a function in the face of heat stress by preventing oxidative damage as co-expression of either SLN or PLN with SERCA2a prevented head-induced carbonylation and nitrosylation of the pump (132). Furthermore, SERCA showed increased susceptibility to thermal damage and inactivation in both $Sln^{-/-}$ and $Pln^{-/-}$ samples compared to WT (132).

While SERCA regulatory proteins have not been examined in the context of SERCA protection with aging, the expression pattern of SLN aligns with the hypothesis that SLN could offer protection for SERCA against age-induced oxidative damage especially in older adult

females. The higher endogenous expression of SLN in the soleus of female mice witnessed in study 3 aligns with data from the literature showing inherent protection against oxidative damage in females compared to males. Furthermore, the dynamic upregulation of SLN content during unloading and disease, states which mimic many features of aging, corresponds with the ability of SLN to stabilize and protect SERCA. In order to examine the role of SLN in SERCA protection with aging between sexes, soleus muscles from male and female, WT and *Sln*$^{-/-}$ animals were assessed for SERCA function and SERCA damage at two age groups: adult (4-6 month) and aged (18+ month). The expression of SERCA1a, SERCA2a and SLN were assessed via western blotting to examine changes with aging between sexes. Both SERCA ATPase activity and SERCA-mediated Ca^{2+} uptake were assessed as markers of SERCA function. Finally, co-immunoprecipitation of SERCA2a and SERCA1a with 3-nitrotryosine (3-ny) was conducted to assess nitrosative damage to SERCA.

4.3 Methods

4.3.1 Animal Model

A continuous breeding colony of *Sln*$^{-/-}$ mice was previously established at the University of Waterloo's Central Animal Facility. Breeding two heterozygous *Sln*-null animals yielded offspring that were wild-type (+/+; WT), heterozygous (+/-), and homozygous (-/-) mice. To differentiate the genotype, all animals were ear notched and RT-PCR was done using DNA taken from the ear notches. For RT-PCR, the sample DNA was combined with GB-AMP™ 2x PCR Master Mix (Genebio Systems, Inc.) and 5’ and 3’ primers for both the wild type (WT) and *Sln*-null (SLN-WT, forward, 5’-TGT CCT CAT CAC CGT TCT CCT-3’and reverse 5’-GCT GGA GCA TCT TGG CTA ATC-3’; *Sln*-null, forward, 5’- GTG GCC AGA GCT TTC CAA). TA-3' and its reverse complement 5'-CAA AAC CAA ATT AAG GGC CA-3'). The samples were then put into a thermal cycler (MJ MINI, Bio-Rad, Canada) and heated for 3 minutes at 94°C, followed by 30 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 54°C, and extension for 60 seconds at 72°C, with a final extension at 72°C for 7 minutes. The amplified products were then separated on a 1% agarose gel containing 0.01% ethidium bromide (BioShop, Canada) and visualized using a ChemiDoc imaging system (Bio-Rad, Canada). For this thesis, experiments were conducted on both male and female littermate wild-type (WT) and *Sln*$^{-/-}$ mice, aged between 4-6 months and 18+ months. All animals were kept in an environment-controlled room with a 12:12 light/dark cycle and given unlimited access to food (Tekland 22/5 Rodent Diet, Harland-Tekland, Madison, WI) and water. The University of Waterloo's Animal Care Committee approved the study, and all procedures were conducted in accordance with the Canadian Council on Animal Care.

4.3.2 Muscle Preparation

All experimental animals were sacrificed via cervical dislocation after which animals were weighed for total body weight and soleus muscles were dissected and weighed. Excised soleus muscle was homogenized using a handheld glass mortar and pestle homogenizer at 10:1 (vol/wt) in ice cold PMSF buffer (250mM sucrose, 5 mM HEPES, 10mM NaN3 and 0.2 mM phenylmethanesulfonyl fluoride, pH 7.5). Homogenates were frozen in liquid nitrogen and stored in the -80°C freezer until use for Western blots, co-immunoprecipitation and SERCA function assays.

4.3.3 Western Blotting

Western Blotting was performed to determine protein expression for SLN, SERCA1a, SERCA2a and nitrosylation. Equal protein quantity of soleus samples, along with laemmli buffer, were loaded into 7.5% (SERCAs & 3-ny) glycine-based polyacrylamide gels and 7.5% tricine based polyacrylamide gels for smaller proteins (SLN). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed in order to separate proteins of interest by size. Once separated, proteins were transferred via wet transfer (except for SERCAs which are semi dry) onto either a nitrocellulose (SLN) or PDFV membrane (all other proteins). Following transfer, the membranes were blocked in either 5% skim milk or 5% BSA in TBST at room temperature for one hour. After blocking, membranes were incubated with primary antibodies overnight at 4°C. Membranes are then washed in TBST, before entering incubation with the respective secondary antibody for one hour at room temperature. Membranes then undergo a final series of washes with TBST before the administration of a chemiluminescence

agent. All western blot imaging was done with a ChemiDoc Imaging System and optical density analysis is completed using Image Lab

4.3.4 Co-Immunoprecipitation

Co-immunoprecipitation was conducted to assess nitrosylation damage to SERCA with aging. Protein G bound to Agarose beads (Santa Cruz Biotechnology, sc-2002) was coupled with 5µg of antibodies directed against SERCA1a, SERCA2a, or 3-ny. Protein G agarose bead-antibody complexes were incubated for one hour at 4°C with 400 µg of protein from the soleus homogenate. After low-pH elution, proteins were solubilized in 5x solubilizing buffer and stored at -80°C until western blot analysis, where membranes were then probed for nitrosylation (3-ny), SERCA1a, or SERCA2a.

4.3.5 SERCA Activity

Soleus homogenates were used to determine Ca^{2+} uptake as described previously. Fluorescence signals produced by the Ca^{2+} fluorophore Indo-1 were collected using a dual-emission wavelength spectrofluorometer. As Indo-1 emits light at different wavelengths when bound (485 nm) or unbound to Ca^{2+} (405 nm), this assay captures the rate of Ca^{2+} uptake by analyzing the change in bound and unbound Indo-1 emissions. Two milliliters of reaction buffer (200 mM KCl, 20 mM HEPES, 10mM NaN_3, 5 µM TPEN, 15mM $MgCl_2$, and 5mM oxalate, pH 7.0 at 37 °C) was added to a cuvette, along with 1.5 µM Indo-1. As opposed to Chapter 3, the addition of oxalate to the SERCA uptake buffer in this study eliminated back inhibition of SERCA through the formation of Ca^{2+}-oxalate complexes. $CaCl_2$ (3µl) was added to achieve an initial [Ca^{2+}] between 3 and 3.5 µM. Prior to the addition of the homogenate, background

data were collected using the Felix software (Photon Technology International, Birmingham, NJ, USA), after which ATP (5mM) was added to initiate Ca^{2+} uptake. Rates of Ca^{2+} uptake were assessed at physiologically relevant free [Ca^{2+}] of 100 nM to 1000 nM. After capturing uptake rates, cyclopiazonic acid, a SERCA inhibitor, was added to the cuvette and the rate of change in fluorescence was measured to determine SR vesicle leak.

Maximal Ca^{2+}-activated SERCA activity (Vmax) was measured using a spectrophotometric plate assay as previously described (241) (209). Briefly, soleus (20ul) homogenate was added to 5 ml of assay buffer (200mM KCl, 20mM HEPES, pH 7.0, 10mM NaN_3, 1mM EGTA, 15mM $MgCl_2$, 10mM phosphoenolpyruvate, 5mM ATP, 18 U/ml of both lactate dehydrogenase and pyruvate kinase, and 1mM Ca^{2+} ionophore A-23187 (Sigma)). Similar to the addition of oxalate in the SERCA uptake buffer, the addition of the Ca^{2+} ionophore A-23187 eliminated back inhibition of SERCA by forming stable complexes with Ca^{2+} and facilitating transport across the SR membrane. The sample buffer mixture was then added to 16 tubes containing varying amounts of Ca^{2+}, resulting in Ca^{2+} concentrations ranging from approximately 7 to 4.5 *p*Ca units. 100ul from each tube will then be loaded onto a 96 well plate in duplicate and 2ul of NADH is used to initiate the ATPase reaction. Background ATPase activity was captured by the addition of the SERCA inhibitor, cyclopiazonic acid (CPA), in a final well, and any basal ATPase activity recorded from this well was subtracted from Vmax. The plate was read at a wavelength of 340 nm for 30 min at 37°C on SpectraMax ABS Plus (Molecular Devices) using SoftMax Pro 7 Software. Upon protocol completion, data were entered into GraphPad Prism, where they were plotted against a negative logarithmic pCa. Vmax was established as the maximal activity, and EC50 (the [Ca^{2+}] required to achieve

50% maximal activity) was determined using a non-linear regression curve fitting using the sigmoidal dose response equation. A subsequent fluorometric plate assay using Indo-1 was used to ensure accurate quantification of [Ca^{2+}] for each data point.

4.3.6 Statistical Analyses

For the comparison of SERCA and SLN content, statistical significance was determined using a two-way ANOVA with sex and age as factors. For all remaining measures in this study, a three-way ANOVA was used to assess the effect of sex, genotype and age. As the primary objective of this study was to examine if SLN played a role in protecting SERCA against age-related oxidative damage, when sex was not a significant factor, data from male and female mice of the same age and genotype were combined and a two-way ANOVA for age and genotype was used. Statistical processing was conducted using the SPSS univariate function (version 25, IBM, Armonk, NY, USA) and graphs were generated with GraphPad Prism (version 7, GraphPad Software Inc., CA). For all study comparisons, statistical significance is set to $p \leqq 0.05$ and when appropriate, a Bonferroni post hoc test was used to compare pairwise means. Values are means ± SEM.

4.4 Results

4.4.1 Expression of SLN and SERCA Isoforms

Western blotting was conducted on soleus muscle homogenate of male and female, adult (4-6M) and older adult (18+M) wild type mice to assess the total protein for SLN, SERCA1a and SERCA2a. For SERCA1a there was a significant main effect of age, wherein older animals displayed lower SERCA1a content compared with adult (Fig. 8A, $p = 0.02$). There were no effects of sex ($p = 0.18$). Conversely, when examining SERCA2a content, there was a significant interaction between sex and aging (Fig. 8B, $p = 0.02$). In young adults, females displayed significantly greater SERCA2a content than age-matched males (0.043 ± 0.021vs. 0.013 ± 0.003, $p = 0.030$). With aging, males displayed a significant increase in SERCA2a content ($p = 0.007$), resulting in statistically similar SERCA2a levels between older adult females and males (0.043 ± 0.014 vs. 0.060 ± 0.031, $p = 0.190$). Finally, there was a significant main effect of both age and sex on SLN content (Fig. 8C, $p = 0.02$ for both age and sex), with females and older animals displaying greater expression of SLN.

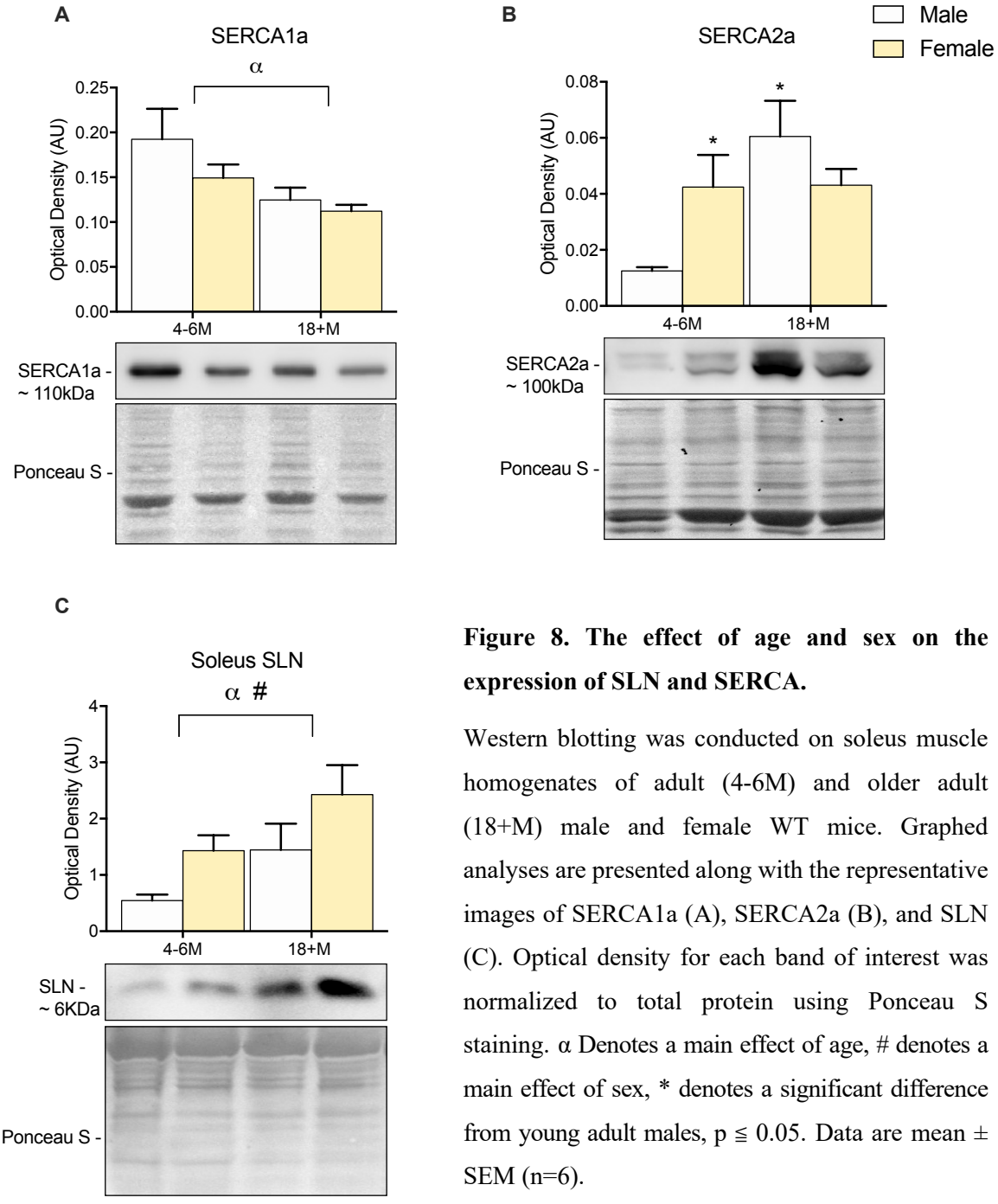


Figure 8. The effect of age and sex on the expression of SLN and SERCA.

Western blotting was conducted on soleus muscle homogenates of adult (4-6M) and older adult (18+M) male and female WT mice. Graphed analyses are presented along with the representative images of SERCA1a (A), SERCA2a (B), and SLN (C). Optical density for each band of interest was normalized to total protein using Ponceau S staining. α Denotes a main effect of age, # denotes a main effect of sex, * denotes a significant difference from young adult males, $p \leqq 0.05$. Data are mean ± SEM (n=6).

4.4.2 SERCA Function

SERCA ATPase activity was measured using a spectrophotometric plate assay across a range of Ca^{2+} concentrations to determine both maximal SERCA ATPase activity (Vmax) and SERCA Ca^{2+} affinity (Fig. 9A). No sex differences were found in either maximal SERCA ATPase activity or the sensitivity of SERCA to Ca^{2+} and as such data from male and females of the same genotype were combined. While genotype did not impact Vmax (Fig. 9B, $p = 0.93$), there was a main effect of age as older adult mice displayed blunted maximal SERCA activity compared to young adult counterparts ($p = 0.05$). EC50 is the Ca^{2+} concentration required to elicit 50% of Vmax and is considered a measure of SERCA Ca^{2+} affinity. While age did not significantly impact EC50 (Fig 9C, $p = 0.65$), there was a main effect of genotype wherein WT animals who express SLN required aa higher [Ca^{2+}] to reach 50% Vmax than *Sln*$^{-/-}$ animals ($p = 0.03$).

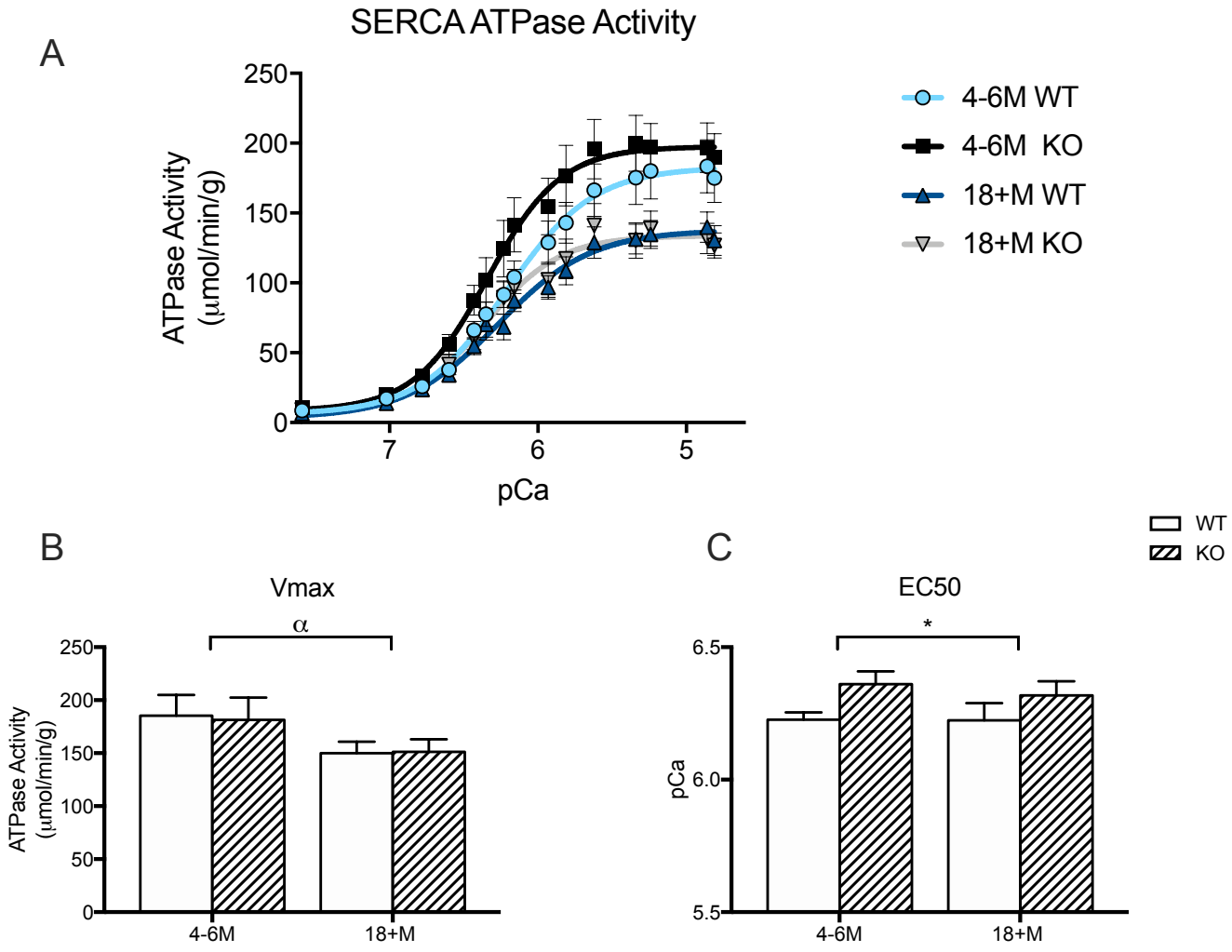


Figure 9. The effects of age and SLN expression on SERCA ATPase activity in soleus muscles.

Ca^{2+}-ATPase activity was analyzed over Ca^{2+} concentrations ranging from *p*Ca 7.6 to *p*Ca 4.8 in soleus muscle homogenates of wild type (WT) and *Sln*$^{-/-}$ (KO) mice at two age groups: young adult (4-6M) and older adult (18+M). Average ATPase activity curves are displayed against a negative logarithm of Ca^{2+} concentrations (A). Vmax is the maximal SR Ca^{2+}ATPase activity expressed as μmol•g protein•min (B). EC50 is the negative log of the Ca^{2+} concentration required to attain the half-maximal Ca^{2+}-ATPase activity rate (C). * Denotes a main effect of genotype, α denotes a main effect of age, $p \leqq 0.05$. Data are mean ± SEM (n=10, 5 male and 5 female).

As transport of Ca^{2+} into the SR can be uncoupled from SERCA ATP hydrolysis, Ca^{2+} uptake was also measured as an indicator of SERCA function. Similar to SERCA ATPase activity, no differences were found in SERCA-mediated Ca^{2+} uptake between sexes and data were combined to assess the impact of age and genotype (Fig. 10A). SLN expression and age did not impact the initial peak Ca^{2+} (Fig. 10B). As [Ca^{2+}] alters SERCA activity, the rate of SERCA-mediated SR Ca^{2+} uptake was assessed at *p*Ca 5.7 (2000 nM), 6.3 (500 nM) and 6.6 (250 nM) (Fig. 10D-F). At all Ca^{2+} concentrations measured, there was a main effect of age on the rate of SR Ca^{2+} uptake as the soleus of older animals displayed blunted uptake rates ($p < 0.05$ for all). SLN ablation did not display a significant main effect on Ca^{2+} uptake at any of the [Ca^{2+}]s measured. Finally, there were no significant differences in SR leak between genotype ($p = 0.26$) or age ($p = 0.95$) groups (Fig. 10C).

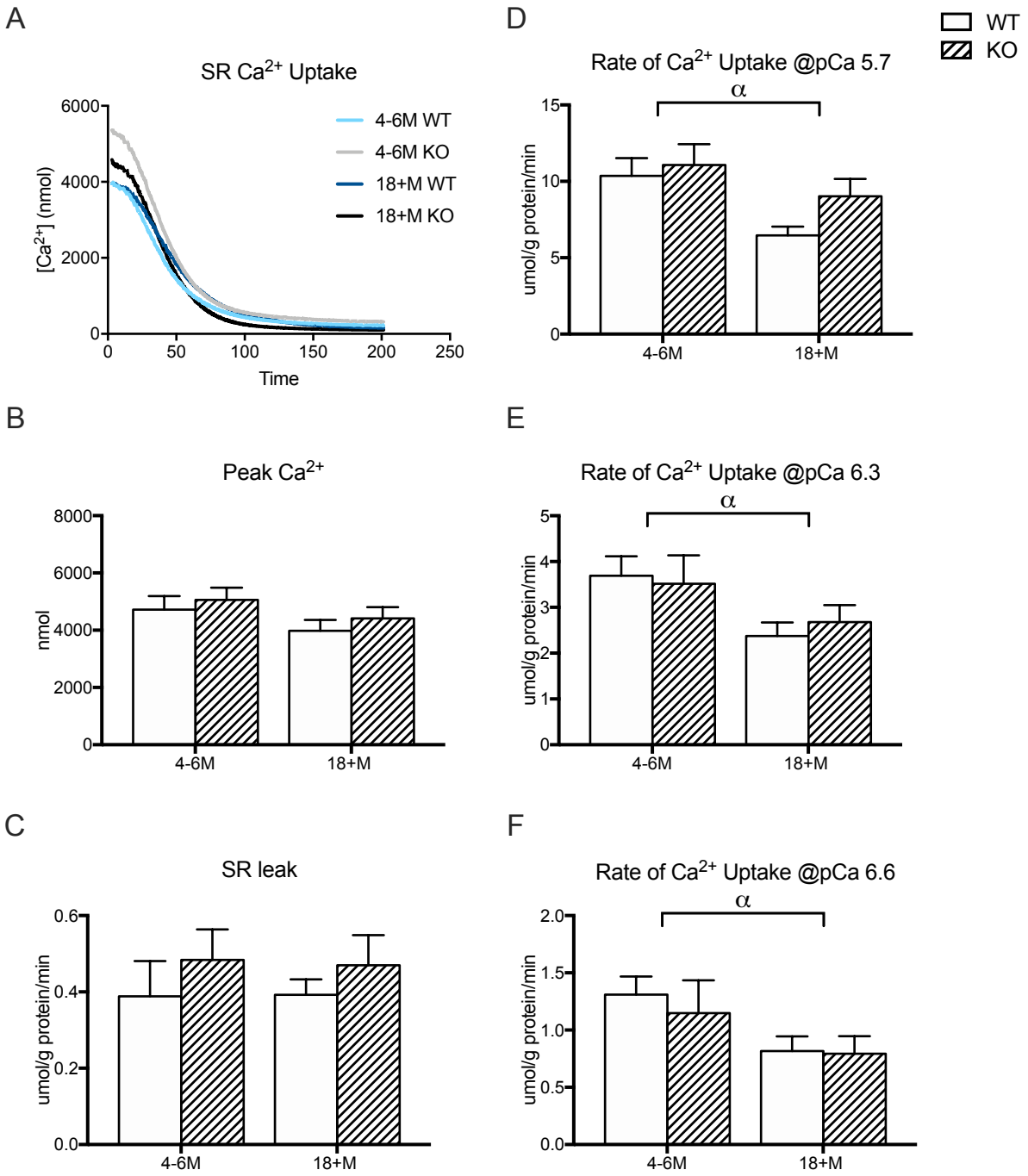


Figure 10. The effect of SLN ablation on SERCA Ca^{2+} uptake and leak in soleus muscles of young and older adult mice.

SERCA-mediated Ca^{2+} uptake was assessed in soleus homogenate isolated from young adult (4-6M) and older adult (18+M) WT and *Sln*$^{-/-}$ (KO) mice (A). Peak Ca^{2+} was captured as the initial [Ca^{2+}] before Ca^{2+} uptake initiated (B). The rate of Ca^{2+} uptake expressed in µmol•g protein•min was measured at *p*Ca 2000 (D), 500 (E), and 250 (F). SR leak was measured as the rate of change following the addition of the SERCA inhibitor, CPA (C). α Denotes a main effect of age, * denotes a significant difference from young adult group of the same genotype, $p \leqq 0.05$. Data are mean ± SEM (n=10, 5 male and 5 female).

4.4.3 The impact of SLN ablation on SERCA damage

In order to examine age related damage to SERCA by RONS, both SERCA1a and SERCA2a were immunoprecipitated and subsequently loaded on polyacrylamide gels for Western blotting to quantify the ratio of the nitrosylation marker, 3-ny, to SERCA content in the immunocomplex (Fig. 11C & D). For both SERCA1a and SERCA2a there was a main effect of age (Fig. 11A SERC1a $p = 0.03$, Fig. 11C SERCA2a $p < 0.001$), wherein soleus muscle from older animals, regardless of sex or genotype, displayed a greater amount of 3-ny bound to SERCA than their younger counterparts. For SERCA1a, there was a main effect of sex with females displaying higher levels of 3-ny co-immunoprecipitated with SERCA1a than males ($p = 0.04$). Conversely, sex was not a main factor for 3-ny & SERCA2a ($p = 0.21$). SLN ablation was not a significant factor for nitrosative damage to either SERCA1a ($p = 0.51$) or SERCA2a ($p = 0.18$).

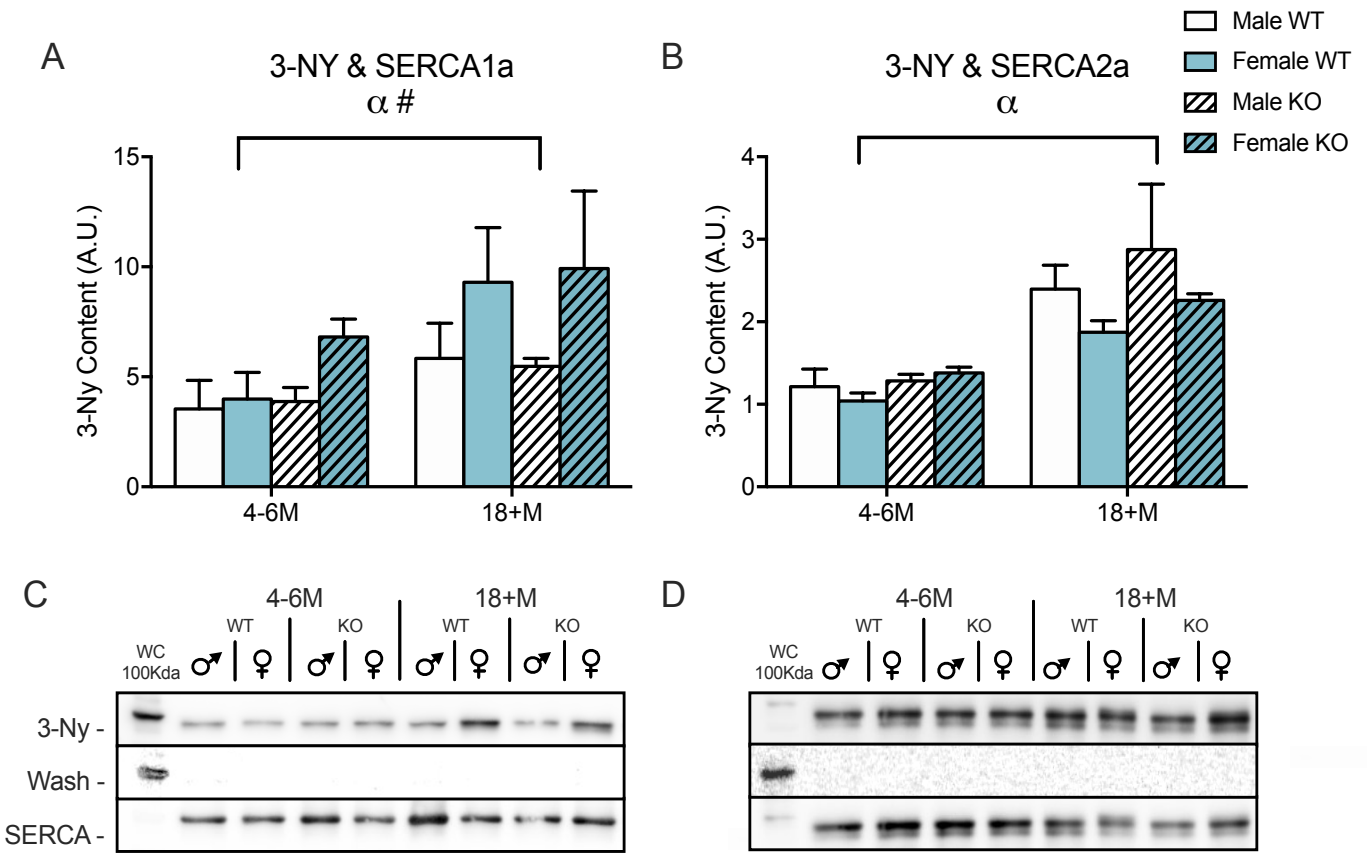


Figure 11. The impact of SLN ablation on SERCA nitrosylation in aging.

Co-immunoprecipitation of SERCA1a (A & C) or SERCA2a (B & D) with 3-Ny was conducted on soleus muscles from male and female, WT and $Sln^{-/-}$ (KO) mice at two age groups: young adult (4-6M) and older adult (18+M). The presence of 3-Ny and SERCA1a or SERCA2a content in the immunocomplex was determined via western blot. Optical density of the 3-Ny band was normalized to the optical density of the respective SERCA band in order to compare nitrosative damage between groups. α Denotes a main effect of age, # denotes main effect of sex, * denotes a significantly difference from the young adult genotype-matched group, $p \leqq 0.05$. Data are mean ± SEM (n=3).

4.5 Discussion

The main objectives of the present study were to investigate how SLN and SERCA content change in both sexes with aging and to discern if SLN played a role in promoting SERCA function by protecting the pump from age-induced RONS damage. With respect to SERCA content, the results of this study show a significant decrease in SERCA1a expression with aging in both sexes. Inverse to SERCA1a, aging resulted in increased SERCA2a content in males only, with no changes observed in females. In accordance with the literature, this study found that aging was associated with reduced SERCA function and increased SERCA damage in both male and female mice. This study also confirmed the findings of previous studies showing that SLN expression is increased with aging and revealed the novel findings that females display greater SLN in both young and older animals. SLN ablation was found to increase the Ca^{2+} affinity of SERCA, regardless of sex and age. However, contrary to the study hypotheses the ablation of SLN did not result in reduced SERCA function or increases to SERCA damage in older animals, suggesting that increase in SLN expression with aging did not offer SERCA protection against age-related oxidative damage.

Previous studies examining SERCA content with aging has predominately been conducted using male animals and has resulted in mixed findings. Similar to this study, others have found reductions in SERCA1a content in male rodent soleus muscle with aging (158, 242), albeit this phenomenon may be exclusive to the slow twitch muscles as those examining fast twitch muscles have found no changes in SERCA content with aging (65, 118, 243). Less research has examined SERCA2a content with aging and while some found no changes (118), Xu et al (2017) found similar results to the present study, with increases in SERCA2a content

in male rat soleus with aging (244). Age-related shifts in the fibre type composition of muscle may account for some of the changes witnessed in SERCA content with aging. Specifically, aging has been associated with a reduction in type II fibres (134) and given SERCA1a is predominately expressed in this fibre type it may account for the reductions witnessed in SERCA1a with aging. Similarly, SERCA2a is predominately expressed in type I fibres and although type I fibre count does not change with age, there is a greater proportion of type I fibres with aging due to the reduction in type II fibres (134). As the same total protein load is used for all samples in a Western blot, this apparent shift to type I fibres with aging would result in greater SERCA2a content. However, in the present study the increase in SERCA2a with aging was exclusively witnessed in males who historically display lower type I fibre counts than their female counterparts (245), suggesting this finding may not be entirely attributed to shifts in fibre type.

While the literature shows mixed results on the impact of aging on SERCA content, studies have consistently found reduced SERCA function with aging (116, 118, 122, 246). The data from this study is in agreement with previous research as both the maximal SERCA ATPase activity and SR Ca^{2+} uptake in soleus samples from older adult mice were reduced compared to young adults. The reduction in SERCA function is likely due in part to damage to the pump by RONS. As mentioned above, it is well established that aging results in increased mitochondrial disfunction, increased RONS production and an imbalance in redox signalling and several studies have shown age-induced oxidative and nitrosative damage to SERCA (116, 118, 119, 122, 158, 239, 246). In the present study, we examined SERCA damage by RONS

using co-immunoprecipitation of both SERCA1a and SERCA2a with 3-ny, finding an increase in nitrosylation to both SERCA1a and SERCA2a with aging.

However, it should be noted that sex impacted the 3-ny content of SERCA. Previous literature has found that males display greater age-related oxidative damage than their female counterparts (200-202). In the present study, this was not found to be the case. For SERCA2a, there was no effect of sex on SERCA2a-3ny content across age groups. Conversely, for SERCA1a, there was a significant main effect of sex when examining SERCA1a-3-ny, wherein females displayed greater nitrosylation of SERCA1a than males, regardless of age. Although the current study did not seek to elucidate the mechanism driving the increased SERCA1a damage in females, examination of sexual dimorphism in the interplay of oxidative metabolism and redox balance within specific fibre types may offer further insight into this finding.

The sex differences in the SERCA1a-3ny data are especially interesting in the context of aging. Previous research has suggested that age-related nitrosylation of SERCA may be exclusive to SERCA2a (111, 118). Specifically, Viner et al (1996) examined isolated SR vesicles of rat hindlimb finding that SERCA2a accumulated nitrotyrosine with aging, while SERCA1a did not (111). A follow up study by Viner et al (1999) similarly found that while both SERCA isoforms show reduced cysteine residues with aging, SERCA2a selectively displayed an age-dependent increase in nitrotyrosine (118). As mentioned above, this study found an increase to SERCA1a-3ny with aging, converse to the Viner papers. While the sequence of SERCA2a is inherently more susceptible to RONS damage in both sexes, the inclusion of females may explain the discrepancies between the current study and the literature

for SERCA1a. Both studies by Viner and colleagues, along with the majority of research examining SERCA oxidative damage with aging, did not included a sex comparison, either exclusively examining male animals (111, 118, 122, 158, 239) or having not reported on sex (116, 119, 246). The finding that SERCA1a-3ny increased with aging in the present may be partially attributable to the inclusion of female mice, given females, especially at the older adult age group, displayed greater SERCA-3ny content. As such, future studies should be sure to include both males and females when examining the susceptibility of SERCA isoforms to age-related damage.

Although recent studies have shown that SLN expression is increased with aging (163, 164, 247), the present study is the first to examine changes to SLN content in both males and females with aging and to investigate the effect of SLN on SERCA function and protection with aging. Consistent with the previous literature (163, 164, 247), this study found that SLN content was greater in the soleus muscles of older adult mice than young adult mice. Likewise, this study shows that the greater SLN content witnessed in the soleus muscles of young adult female mice in Chapter 3, persists throughout aging. The previous studies which found SLN upregulation with aging speculated that increases in SLN expression may contribute to the reduced SERCA function, Ca^{2+} dysregulation and worsened muscle function witnessed with aging. However, studies examining the endogenous upregulation of SLN in atrophic unloading and muscle disease suggest that the increase in SLN is beneficial as ablation of SLN resulted in reductions to SERCA function, greater losses in muscle mass and worsened disease markers (66, 98, 101). Furthermore, recent research has shown that SLN is capable of protecting SERCA from RONS damage and thermal inactivation in the face of heat stress (132). Given

this additional information, in the present study it was hypothesized that SLN would be upregulated with aging in order to protect SERCA from age-induced oxidative damage.

Contrary to the initial hypothesis, the present study found little evidence that SLN offers SERCA protection from RONS damage with aging. When examining SERCA damage, there were no significant differences found in the amount of 3-ny bound to either SERCA isoform between WT and *Sln*$^{-/-}$ animals. Furthermore, if SLN offered SERCA protection from age-induced oxidative damage, one would expect to see worsened SERCA function in aged *Sln*$^{-/-}$ animals compared to their age matched WT counterparts which was not witnessed in this study. Akin to the studies previously conducted on young adult male mice (57), SLN ablation had no impact on maximal ATPase activity in either age group and improved the Ca^{2+} affinity of SERCA, regardless of age. However, it should be noted that while aging was a statistically significant factor in reducing rates of SERCA-mediated Ca^{2+} uptake, post hoc analyses revealed that reductions in the rate Ca^{2+} uptake with aging only reached significance between young adult and older adult WT animals. This finding suggests that WT animals who express SLN show greater losses in SERCA-mediated Ca^{2+} uptake with aging than animals who lack SLN which aligns with the notion put forth by previous investigators that the upregulation of SLN with aging is maladaptive. However, ablation of SLN did not significantly improve maximal SERCA function or result in lessened SERCA damage and future research examining the direct impact of SLN on Ca^{2+} transients and muscle function would be required in order to speculate on the maladaptive or beneficial nature of the upregulation in SLN with aging.

Alternatively, the data from this study may be more indicative of the uncoupling capacity of SLN. Previous research has shown that SLN can uncouple SERCA ATPase activity from Ca^{2+} uptake (45, 46) and given the upregulation of SLN witnessed with aging, it would be expected to cause greater disparities in Ca^{2+} transport in relation to ATPase activity between aged genotype groups. Moreover, SLN has been shown to be dynamically recruited in order to promote energy expenditure and heat production. Specifically, SLN expression has been shown to increase in response to a high fat diet and ablation of SLN results in lower whole body metabolic rate during the animal's waking active hours (57). As changes to body composition, mainly an increase in adiposity, is witnessed with aging, SLN could be similarly recruited in an effort to promote energy expenditure with age-induced obesity and future studies should examine the role of SLN in metabolic expenditure with aging.

In conclusion, the primary objectives of this study were to examine alterations to the content of SERCA and SLN in response to sex and aging, while also examining the potential protective role of SLN against age-induced RONS damage to SERCA. The results presented here are consistent with previous work showing that SLN is greater in females than males and is upregulated with aging. Contrary to the initial hypothesis, SLN does not appear to confer significant protection against oxidative stress to SERCA during the aging process, however this study revealed novel sex-dependent disparities in the oxidation of differing SERCA isoforms which may contradict the current notion that SERCA2a is more susceptible to age-induced oxidative damage. The current study emphasizes the need for the inclusion of both sexes in future studies examining SERCA and SLN and further investigation into how upregulations in SLN may impact Ca^{2+} homeostasis, metabolism and muscle function in aging.

Chapter 5: The Impact of SLN Ablation on Cn Signaling and Skeletal Muscle Phenotype and Function with Aging and Sex

5.1 Abstract

Recent research has implicated sarcolipin (SLN), a small protein inhibitor of SERCA, in the activation of calcineurin (Cn), a Ca^{2+} activated phosphatase. In fact, several studies have shown that SLN is dynamically upregulated to activate Cn signalling, promoting greater muscle mass in models of muscle disease and unloading. As aging displays several shared characteristics to both unloading and muscle disease, this study sought to examine how changes to SLN content impact Cn signalling and muscle health with aging in both male and female mice. In the present study, SLN content in the slow-twitch soleus muscle was shown to be greater in females than males and in aged animals compared to young adults. While neither age or sex effected SERCA1a content, aging resulted in an increase in SERCA2a expression which was exclusive to males. As expected, both age and sex impacted muscle fibre type, mass and function. Specifically, females and older animals generally displayed fewer type II fibres, lower muscle mass and reduced force generating capacity than their male and young adult counterparts. To date, the effects of age on Cn activity is unknown. Here, Cn signalling was higher in older adult muscle with no change to total Cn content. Contrary to the hypothesis, the ablation of SLN in aged animals had no impact on Cn signalling. Likewise, there were no differences in muscle fibre type, markers of muscle mass or muscle contractile function with SLN ablation, further suggesting that SLN does not activate Cn with aging. Interestingly, although SLN did not impact Cn signalling, SLN ablation was shown to significantly increase the total number of type I fibres, a finding which is incongruent with the role of Cn-NFATc1 in the promotion

of MHCI. Similarly, this study points to a novel sex difference in the role of SLN in muscle function. Historically, SLN ablation has been shown to increase relaxation rate and to reduce twitch force generation. Findings from this study suggest that the effects of SLN on muscle relaxation and twitch force may be greater in males than females, potentially due to disparities in fibre type. In conclusion, this study adds to the body of literature displaying sex and age-related changes to muscle mass and function and offers the novel findings that although SLN does not promote hypertrophic Cn signalling, both SLN and Cn activity are greater with aging.

5.2 Introduction

The sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) is a vital SR membrane protein responsible for pumping intracellular Ca^{2+} into the SR, eliciting muscle relaxation and maintaining Ca^{2+} homeostasis. Sarcolipin (SLN), a small protein localized within the SR membrane, has emerged as a key regulator of SERCA function. While other SERCA protein regulators have been identified, SLN is unique in its ability to affect both the Ca^{2+} affinity of SERCA and SERCA efficiency (45). More specifically, in the presence of SLN, SERCA displays lower levels of ATPase activity for a given Ca^{2+} concentration and requires greater ATPase activity in order to transport the same amount of Ca^{2+} (22, 45). As SLN disrupts the uptake of Ca^{2+} into the SR by slowing SERCA activity, it can amplify cytosolic Ca^{2+} transients. Overexpression of SLN in myocytes and myotubes has been shown to prolong Ca^{2+} transients and relaxation rate (64, 248). Similarly in mouse soleus, the presence of SLN results in an amplified cytosolic Ca^{2+} transient and increased muscle force production at low frequency stimulations (22). Conversely at higher frequency stimulations, where saturating Ca^{2+} levels persist, SLN does not impact force production (22), collectively suggesting that SLN may have a greater impact on elevating submaximal Ca^{2+} transients.

As the interaction between SERCA and SLN impacts cytosolic [Ca^{2+}], the presence of SLN or changes to SLN content could result in muscle adaptation via the activation of Ca^{2+} signalling. While Ca^{2+} is a potent and varied cell signalling molecule, calcineurin (Cn) may be specifically activated by increases in SLN content. Cn is a Ca^{2+}-sensitive phosphatase activated in response to sustained increases in submaximal intracellular [Ca^{2+}], which SLN has been shown to increase (249, 250). Upon activation, Cn dephosphorylates the transcription factor

NFAT (nuclear factor of activated T cells) family (NFATc1-c4 and NFAT5), allowing their translocation to the nucleus and subsequent changes in gene expression (71). This pathway has been implicated in promoting muscle hypertrophy, fiber-type switching, and other adaptive responses (69, 72, 85). With respect to muscle fibre type, the activation of NFAT isoforms c1-c4 have been associated with promoting MHC transcriptional activity, leading to the expression of genes associated with both slow-twitch and fast-twitch muscle fibers (69). While the interplay between NFAT isoforms in promoting specific MHC variants is complex and not yet fully established, NFATc1 has emerged as the main variant in the promotion of MHCI and slow-twitch phenotype (78).

In addition to MHCI promotion, activated NFATs can stimulate the expression of genes associated with muscle growth. Specifically, activated Cn has been shown to promote the expression of myogenic transcription factors, myogenin (79), myocyte enhancing factor 2 (MEF2) and MyoD (81), and has been implicated in the IGF-1 hypertrophy pathway (83, 84). Together, the activation of these genes promote hypertrophic responses by stimulating muscle differentiation and enhancing the synthesis of contractile proteins. Furthermore, Cn may promote the maintenance of muscle mass through the suppression of muscle atrophy. Indeed, activated Cn has been shown to suppress the expression of myostatin, a negative regulator of muscle growth (82). Additionally, Cn indirectly influences muscle mass by inhibiting the transcription factor FoxO, which regulates the expression of atrophy-related genes (251). Inhibition of FoxO by a constitutively active form of Cn prevented atrophy in myotubes and resulted in enhanced muscle regeneration, reduced scar/fibrotic infiltration and accelerated resolution of inflammation post injury in murine muscle (251). Therefore, Cn activation

orchestrates a multifaceted signaling network that favors muscle protein synthesis and mitigates protein degradation, ultimately promoting the growth and maintenance of skeletal muscle mass.

The initial studies which characterized SLN ablation in mice did not assess Cn signalling and reported no alterations to muscle mass or fibre type between wild type (WT) and SLN ablated ($Sln^{-/-}$) animals (22, 208). While this may suggest that SLN does not play a role in activating Cn basally, the initial studies exclusively examined young adult male animals which display relatively low levels of SLN and subsequent studies have found that the expression of SLN is dynamically regulated during times of cell adaptation. In fact, upregulation of SLN expression has been demonstrated in mechanical unloading (66), unloading due to spaceflight (95), and numerous muscle disease states such as Duchenne muscular dystrophy (DMD) and myotonic dystrophy (98, 101, 102, 108). Several studies have examined the role of this increased SLN content with respect to Cn signalling. Specifically, SLN ablation resulted in reduced Cn signalling along with greater atrophy and exacerbation of disease phenotypes in studies examining mechanical unloading (66), DMD (101) and centronuclear myopathy (98). Together this suggests that in response to muscle stress, SLN is upregulated, at least in part, to promote hypertrophic Cn signalling.

Aging shares many characteristics with unloading and muscle disease, including those impacted by Cn signalling. Like unloading and muscle disease, muscle atrophy is a common feature with aging, with declines in muscle mass starting at 30 years of age and increasing in rate post 60 years of age in humans (252). Similarly, changes to the fibre type profile of skeletal muscle are also common between these three conditions. In unloading conditions,

reductions in chronic muscle activation reduce Ca^{2+} signalling creating a shift towards type II fibres (66, 95). Conversely, in muscle disease and aging, increased resting $[Ca^{2+}]_{cyt}$ is often witnessed with an apparent shift towards type I fibres (145, 253, 254). Furthermore, recent studies (122, 163), including the previous chapter of this thesis, have found that SLN is dynamically upregulated in aging as it is with muscle unloading and disease. Interestingly, while Cn signalling impacts both muscle mass and fibre type and has been shown to be activated by increased SLN during times of muscle stress, changes to Cn content and signalling with aging have yet to be examined in skeletal muscle. As such, this study sought to determine how increases to SLN content with aging impact Cn signalling and the downstream impact on muscle mass, fibre type and overall muscle function in both male and female mice.

5.3 Methods

5.3.1 Animal Model

Previously, a continuous breeding colony of *Sln*$^{-/-}$ mice was established at the Central Animal Facility at the University of Waterloo. The breeding of two heterozygous SLN null animals results in offspring of wild type (+/+; WT), heterozygous (+/-) and homozygous (-/-) mice. In order to distinguish genotype, all animals were ear notched and RT- PCR was conducted using the DNA extracted from said ear notches. To preform RT-PCR, the sample DNA was added to GB-AMP™ 2x PCR Master Mix (Genebio Systems, Inc.) along with 5' and 3' primers for both WT and *Sln*-null (*Sln*-WT, forward, 5'-TGT CCT CAT CAC CGT TCT CCT-3'and reverse 5'-GCT GGA GCA TCT TGG CTA ATC-3'; *Sln*-null, Forward, 5'- GTG GCC AGA GCT TTC CAA TA-3'and reverse 5'-CAA AAC CAA ATT AAG GGC CA-3'). Samples were then placed in a thermal cycler (MJ MINI, Bio-Rad, Canada) and denatured for 3 min at 94°C followed by 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 54°C, and extension for 60 sec at 72°C, followed by a final extension at 72°C for 7 min. The amplified products were then separated on a 1% agrose gel containing 0.01% ethidium bromide (BioShop, Canada) and identified using a Chemidoc imaging system (Biorad, Canada). For the purpose of this thesis, experiments were conducted on littermate WT and Sln$^{-/-}$ mice, both male and female at the ages of 4-6 months and 18+ months. All animals were housed in an environmentally controlled room with a standard 12:12 light/dark cycle and allowed access to food (Tekland 22/5 Rodent Diet, Harland-Tekland, Madison, WI) and water ad libitum. The study was approved by the Animal Care Committee at the University of Waterloo and all procedures were performed in accordance with the Canadian Council on Animal Care.

5.3.2 Muscle Preparation

All experimental animals were sacrificed via cervical dislocation after which animals were weighed for total body weight and soleus muscles were dissected and weighed. Unless otherwise specified, excised soleus muscles were homogenized using a handheld glass mortar and pestle homogenizer at 10:1 (vol/wt) in ice cold PMSF buffer (250mM sucrose, 5 mM HEPES, 10mM NaN3 and 0.2 mM phenylmethanesulfonyl fluoride, pH 7.5). Homogenates were frozen in liquid nitrogen and stored in -80 freezer until use for western blots.

5.3.3 Whole Muscle Contractility

Whole muscle contractility was conducted on a subset of soleus muscles to examine muscle function. Soleus muscles were carefully excised tendon to tendon and mounted in a bath with Tyrodes Buffer (95% O_2, 5% CO_2) containing 121 mM $NaCl_2$, 5 mM KCl, 24 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 5.5 mM glucose, 0.1 mM EDTA, and 0.5 mM $MgCl_2$, pH 7.3, and was maintained at 25°C. Soleus muscles were electrically stimulated via flanking platinum electrodes driven by a biphasic stimulator (Model 710B, Aurora Scientific, Inc) and electrically evoked muscle force was assessed across a range of stimulation frequencies from 1 to 100 Hz. Data was collected using Dynamic Muscle Control Data Acquisition software (Aurora Scientific, Inc) and analyzed in excel in order to determine peak absolute isometric force amplitude (mN), peak isometric force normalized to cross-sectional area (mN/mm^2), and the maximal rates of force development (+dF/dt) and relaxation (-dF/dt).

5.3.4 Histological and Immunofluorescence Imaging

Another subset of soleus muscles was used for histological and immunofluorescent staining protocols. These soleus muscles were excised and mounted in O.C.T. compound (Tissue-Tek) by immersing in isopentane cooled in liquid nitrogen. Samples were then cut in 10-micron thick sections and mounted onto slides for staining. Histology, including hematoxylin and eosin, and Van Gieson, was conducted to assess total fibre count, fibrosis and general muscle health. Imaging histological stains was conducted using Cytation 5 Cell Imaging Multi-Mode Reader (BioTek) and analyzed using Image-J. Immunofluorescence was conducted to assess fibre type as previously described (32)**.** Briefly, slides were blocked in 5% goat serum and PBS, then slides were incubated overnight in blocking serum with primary antibodies for MHCI, MHCIIa, and MHCIIb (Developmental Studies Hybridoma Bank). Excess primary was removed, and the slides were washed in PBS three times for five minutes. Slides were then incubated in blocking serum and corresponding secondary antibodies for one hour after which excess secondary was removed and Prolong Antifade Gold and coverslips were added to the sections. For immunofluorescence, imaging was conducted via an Axio Observer Z1 fluorescent microscope equipped with standard red, green, blue filters, an AxioCam HRm camera, and AxioVision software (Carl Ziess). Quantification of fibre type and cross-sectional area (CSA) was performed using ImageJ software.

5.3.5 Western Blotting

Western Blotting was performed to determine protein expression for SLN, SERCA1, SERCA2a, Cn, NFAT, and NFAT-p. Equal protein quantity of soleus samples, along with lamelli buffer, will be loaded into 7.5% (SERCAs, Cn and NFATs) glycine-based

polyacrylamide gels and 7.5% tricine based polyacrylamide gels for smaller proteins (SLN). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed in order to separate proteins of interest by size. Once separated, proteins were transferred via wet transfer (except for SERCAs which are semi-dry) onto either a nitrocellulose (SLN) or PDFV membrane (all other proteins). Following transfer, the membranes were blocked in either 5% skim milk or 5% BSA in TBST at room temperature for one hour. After blocking, membranes were incubated with primary antibodies overnight at 4°C. Membranes were then washed in TBST, before entering incubation with the respective secondary antibody for one hour at room temperature. Membranes then underwent a final series of washes with TBST before the administration of a chemiluminescence agent. All western blot imaging was done with a ChemiDoc Imaging System and optical density analysis was completed using Image Lab.

5.3.6 Statistical Analyses

For the comparison of SLN content, statistical significance was determined using a two-way ANOVA with sex and age as factors. For all remaining measures in this study, a three-way ANOVA was used to assess the effects of sex, genotype and age. As the primary objective of this study was to determine if SLN was recruited to promote Cn signalling with aging, when sex was not a significant factor, data from male and female mice of the same age and genotype were combined and a two-way ANOVA for age and genotype was used. Statistical processing was conducted using the SPSS univariate function (version 25, IBM, Armonk, NY, USA) and graphs were generated with GraphPad Prism (version 7, GraphPad Software Inc., CA). For all study comparisons, statistical significance is set to $p \leqq 0.05$ and when appropriate, a Bonferroni post hoc test was used to compare pairwise means. Values are means ± SEM.

5.4 Results

5.4.1 SERCA and SLN expression

As in Chapter 4, Western Blotting was conducted on soleus muscle homogenates to discern how age and sex impacted the expression of SLN. Similar to Chapter 4, this study found a significant main effect of both age ($p = 0.018$) and sex ($p = 0.019$) on SLN expression (Fig. 12A & B), wherein females had higher SLN content than males and older animals had greater expression than young adult animals. Age and sex did not significantly impact the expression of SERCA1a (Fig. 12C & D). Conversely, when examining SERCA2a there was a main effect of age ($p = 0.045$), with older animals displaying increased SERCA2a content (Fig. 12E & F). However, it should be noted that there was a strong trend for a sex by age interaction ($p = 0.052$) suggesting that significantly greater SERCA2a content with aging was primarily witnessed in males. When examining post hoc comparisons between age groups, aged male *Sln*$^{-/-}$ animals displayed a significant increase in SERCA2a compared to young adult male *Sln*$^{-/-}$ (0.033 ± 0.01 vs. 0.012 ± 0.01, $p = 0.025$) and aged WT male mice displayed a trend towards increased SERCA2a (0.026 ± 0.01 vs. 0.010 ± 0.01, $p = 0.088$), while female groups did not show significant differences between groups (WT: 0.019 ± 0.01 vs. 0.014 ± 0.01, $p = 0.582$, *Sln*$^{-/-}$: 0.015 ± 0.01 vs 0.021 ± 0.01, $p = 0.537$). The genetic ablation of SLN had no impact on either SERCA1a ($p = 0.999$) or SERCA2a expression ($p = 0.481$).

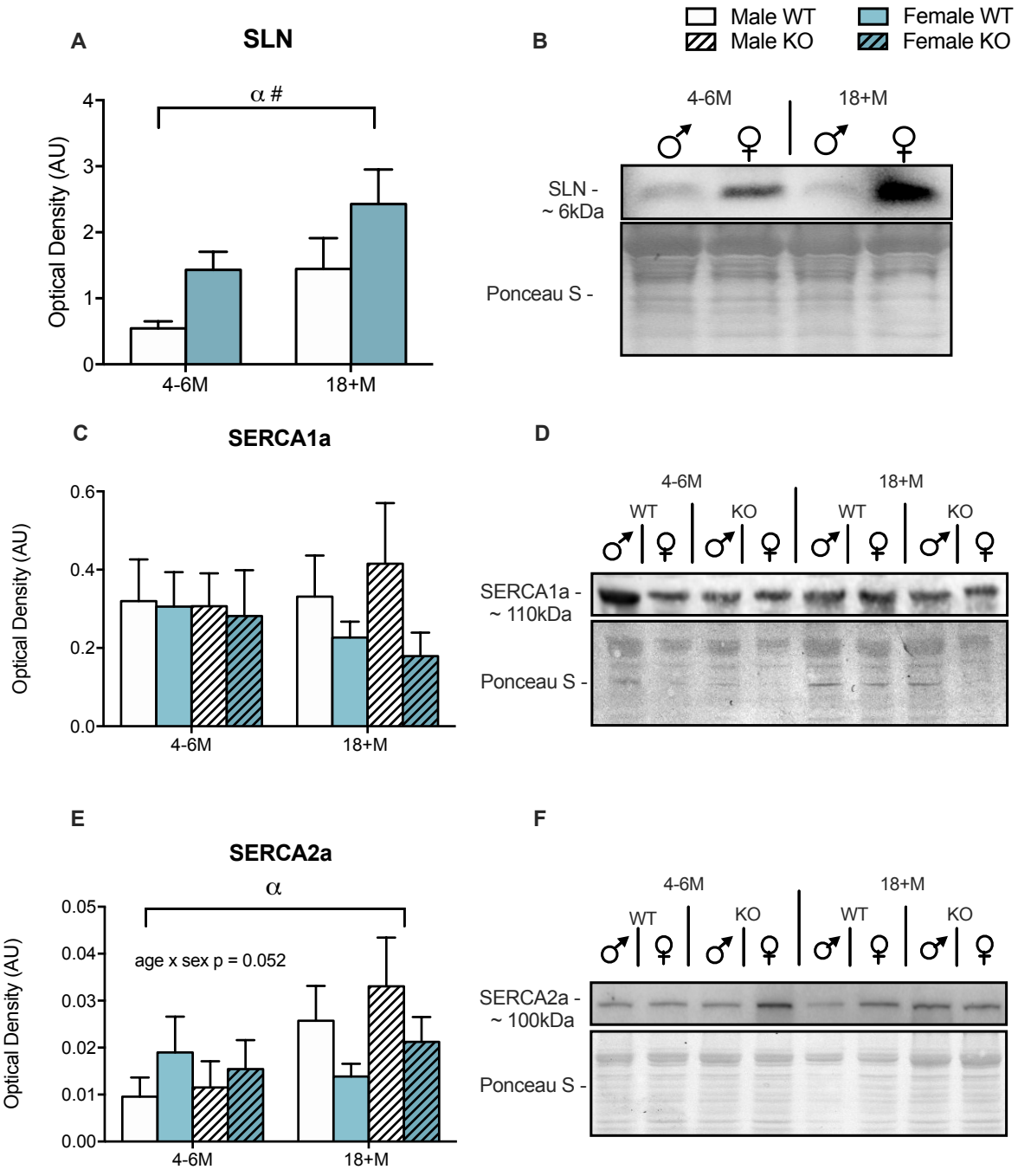


Figure 12. Changes to SERCA and SLN content with sex and aging

Western blotting for was conducted on soleus muscle homogenates of adult (4-6M) and older adult (18+M) male and female WT and *Sln*$^{-/-}$ (KO) mice to determine the content of SERCA and SLN. Graphed analyses of SLN (A), SERCA1a (C) and SERCA2a (E) are presented along with representative blot images and the associated Ponceau S stain (B, D & F). Optical density for each band of interest was normalized to total protein using Ponceau S staining. α denotes a main effect of age, # denotes a main effect of sex, $p \leqq 0.05$. Data are mean ± SEM (n=6).

5.4.2 Cn Signaling

To examine whether SLN activates Cn signalling with aging, Western blotting for both total Cn and the phosphorylation state of NFATc1 was conducted on soleus homogenates of young adult and older adult, male and female, WT and $Sln^{-/-}$ mice. Sex was not a significant factor in either Cn content or activation status of NFATc1 and as such data from male and female mice were grouped in order to better examine the effect of SLN with aging (sex-stratified data available in Appendix A). When examining total Cn content (Fig. 13A & B), SLN ablation had no impact on Cn content ($p = 0.343$) and while the older adult groups on average displayed 21.7% less Cn content than the young adult groups, these differences were not statistically significant ($p = 0.093$). Conversely, age was a significant factor in total NFATc1 content with older animals displaying increased content compared to younger animals (Fig. 13C & F, $p < 0.001$). Age and SLN ablation were not significant factors when examining the amount of phosphorylated, inactive NFATc1 (Fig. 13D & F, age $p = 0.290$, genotype $p = 0.301$). To determine the activation status of NFATc1, the ratio of inactive NFATc1-p to total NFATc1 was examined (Fig. 13E). Here it was seen that aging significantly reduced the NFATc1-p/NFATc1 ratio ($p = 0.003$), while genotype had no effect on the NFATc1-p/NFATc1 ratio ($p = 0.109$).

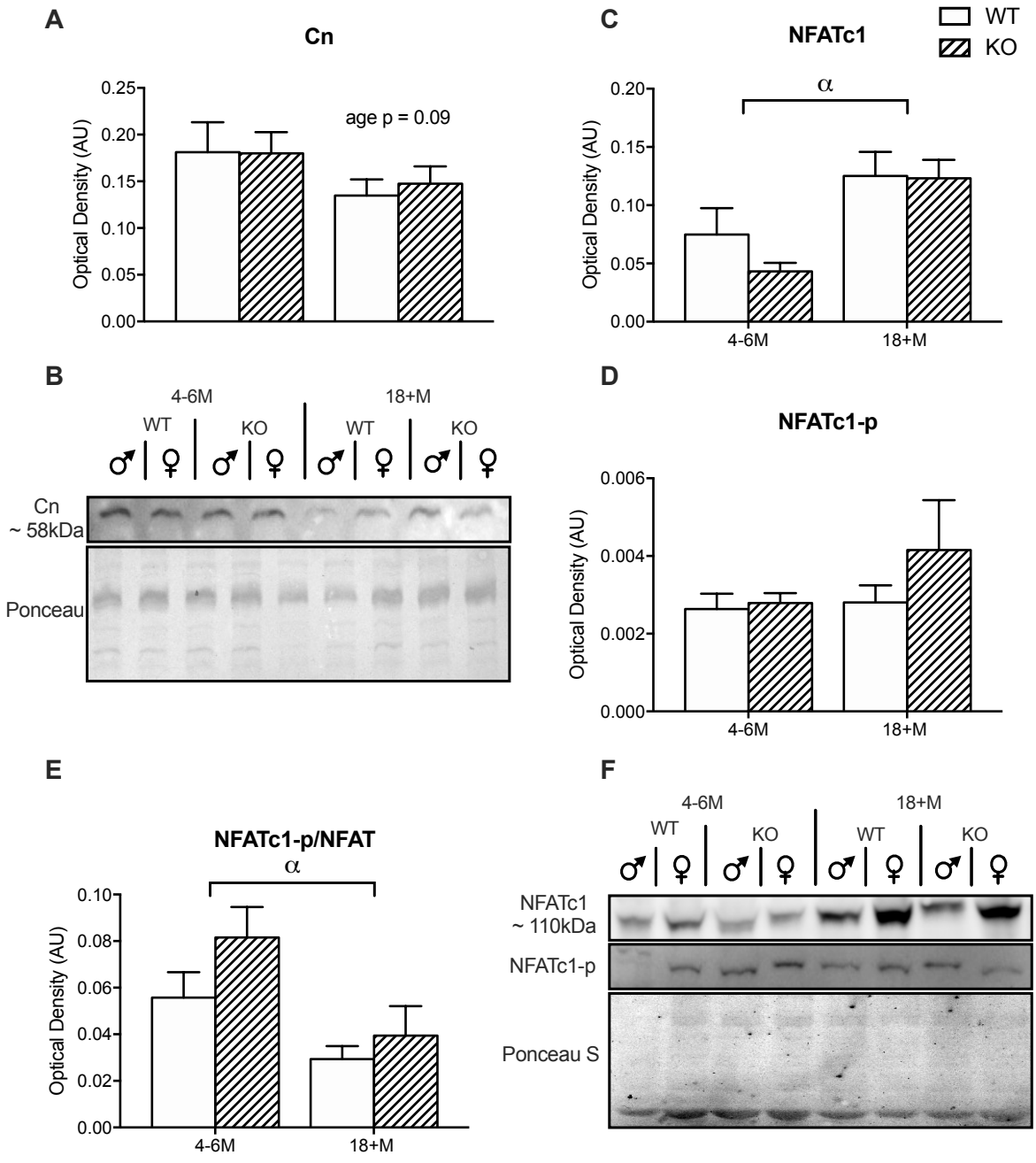


Figure 13. The impact of SLN ablation on Cn signalling with aging

Western blotting for Cn and phosphorylation state of NFAT was conducted on soleus muscle homogenates of adult (4-6M) and older adult (18+M), male and female, WT and *Sln*$^{-/-}$ (KO) mice. Graphed analyses of total Cn (A), NFATc1(C) and NFATc1-p (D) are presented with representative blots and Ponceau S images (B & F). Optical density for bands of interest were normalized to total protein using Ponceau S staining. The ratio of phosphorylated to total NFATc1 (E) was used to determine Cn activity. α denotes a main effect of age, $p \leqq 0.05$. Data are mean ± SEM (n=12, 6 male and female).

5.4.3 Fibre Type

As the downstream target of Cn, NFATc1, is directly implicated in promoting the expression of type I fibre genes, we examined fibre type in the soleus muscles of male and female, young adult and older adult WT and *Sln*$^{-/-}$ mice. When examining the total number of type I fibres, a sex by age interaction reached near significance ($p = 0.053$). In young animals, the type I fibre count was similar between males and females of the same genotype (WT: $p = 0.693$, KO: $p = 0.380$). Conversely, older adult females displayed greater type I fibre counts than their male counterparts, with statistically significant differences between sexes in the both WT (282.2 ± 89.71 vs. 168.2 ± 25.41, $p = 0.032$) and *Sln*$^{-/-}$ groups (363.2 ± 51.65 vs. 248.2 ± 116.05, $p = 0.030$). Similarly, genotype was also a significant factor in type I fibre count ($p = 0.024$), with *Sln*$^{-/-}$ animals generally displaying greater type I fibre counts. When examining the type I fibre count as a percentage of the total number of fibres (Fig. 14C), sex was a statistically significant factor ($p < 0.001$) and the sex by age interaction lessened ($p = 0.084$). Conversely, genotype was no longer significant for type I fibres when presented as a proportion of the total fibre count ($p = 0.430$).

With respect to type II fibres, both sex ($p = 0.034$) and aging ($p = 0.046$) were statistically significant when examining total fibre count (Fig. 14B). While females and older animals generally displayed fewer type II fibres, no specific pairwise comparisons between sex or age groups reached statistical significance. When type II fibre counts were normalized to total fibre count (Fig. 14D), there was a main effect of sex ($p < 0.001$) and a significant interaction between sex and aging ($p = 0.049$). In young adult groups there was no statistical differences between male and female mice, of either genotype (WT: $p = 0.344$, *Sln*$^{-/-}$: $p =$

0.266), but aging resulted in a reduction to the proportion of type II fibres in females compared to their age and genotype matched male counterparts (WT: 0.486 ± 0.100 vs 0.321 ± 0.057, p = 0.007, *Sln*$^{-/-}$: 0.484 ± 0.148 vs 0.293 ± 0.141, p = 0.002).

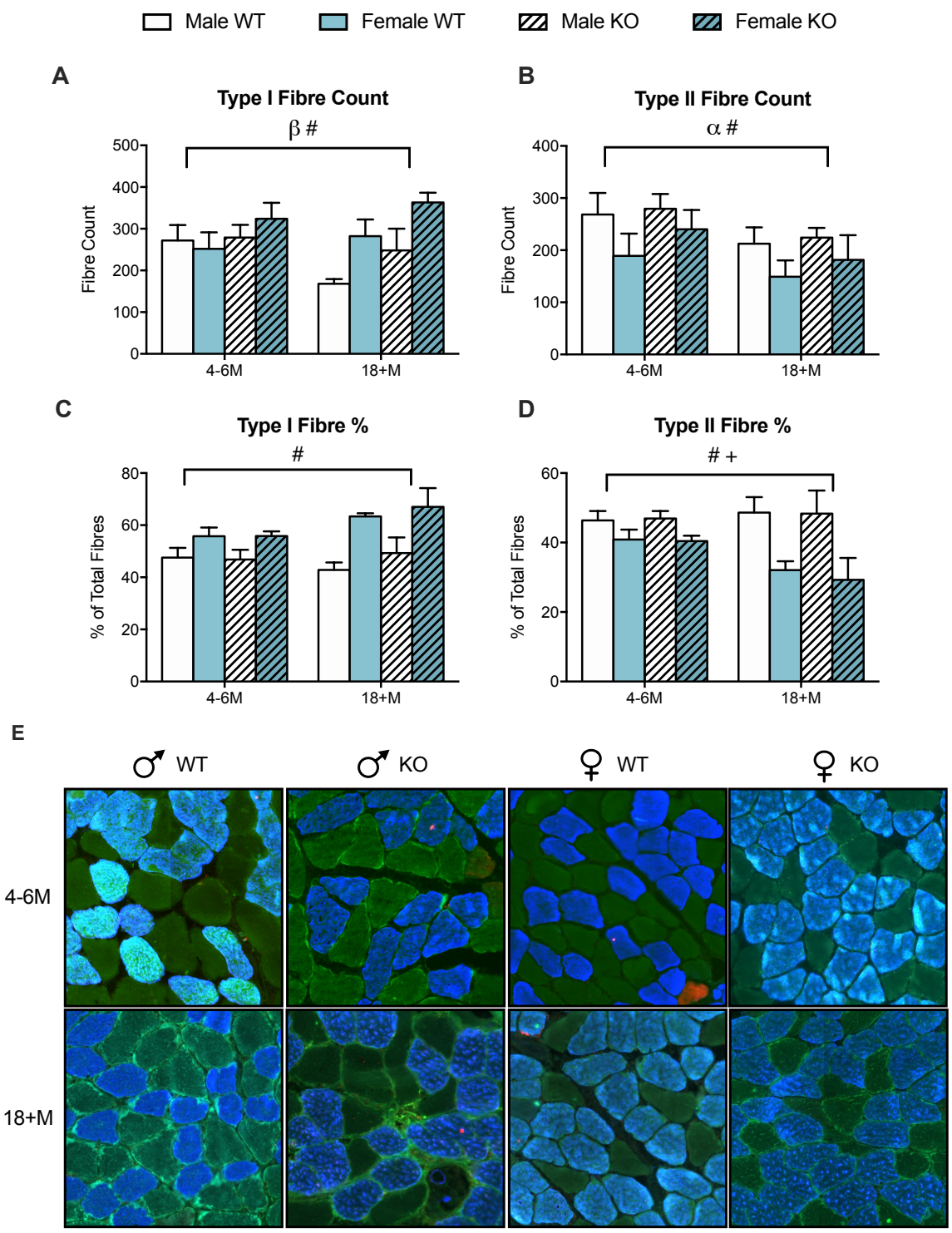


Figure 14. The impact of SLN ablation on muscle fibre type with aging in male and female mice

MHC Immunofluorescent imaging was conducted on soleus muscles from adult (4-6M) and older adult (18+M) male and female WT and *Sln*$^{-/-}$ (KO) mice. Total fibre counts and distribution for both type I (A & C) and type IIA fibres (B & D) were examined. Representative fibre type immunofluorescent images of soleus sections (E) with type I fibres are presented in blue and type IIa in green. α denotes a main effect of age # denotes a main effect of sex, ß denotes main effect of genotype + denotes age by sex interaction, $p \leq 0.05$. Data are mean ± SEM (n=5).

5.4.4 Muscle Mass

To examine changes to muscle mass with SLN ablation during aging, muscle fibre cross sectional area, muscle fibrosis, total fibre counts and whole soleus muscle weights were determined. Here, it was found that sex ($p < 0.001$), but not age ($p = 0.419$) or genotype ($p = 0.517$), was a significant factor in whole soleus muscle weight (Fig. 15A), females displaying lower muscle mass than male genotype-matched counterparts. However, when muscle mass was normalized to body weight (Fig. 15B), the only significant factor was age ($p < 0.001$) with older animals having lower muscle/body weight ratios. There were no statistically significant changes when examining the total number of soleus muscle fibres (Fig. 15C) or the cross-sectional area of type I fibres (Fig. 15D). Conversely, both sex and age affected the cross-sectional area of type II fibres (Fig. 15E, $p = 0.010$ and $p = 0.012$, respectively), with males and older animals generally displayed larger type II fibre CSA. Finally, muscle fibrosis was assessed as a measure of muscle quality (representative images of fibrosis available in Appendix B). There was a significant main effect of age for muscle fibrosis (Fig, 15F, $p < 0.001$), with statistically significant increases in fibrosis with aging in all sex and genotype specific pairs.

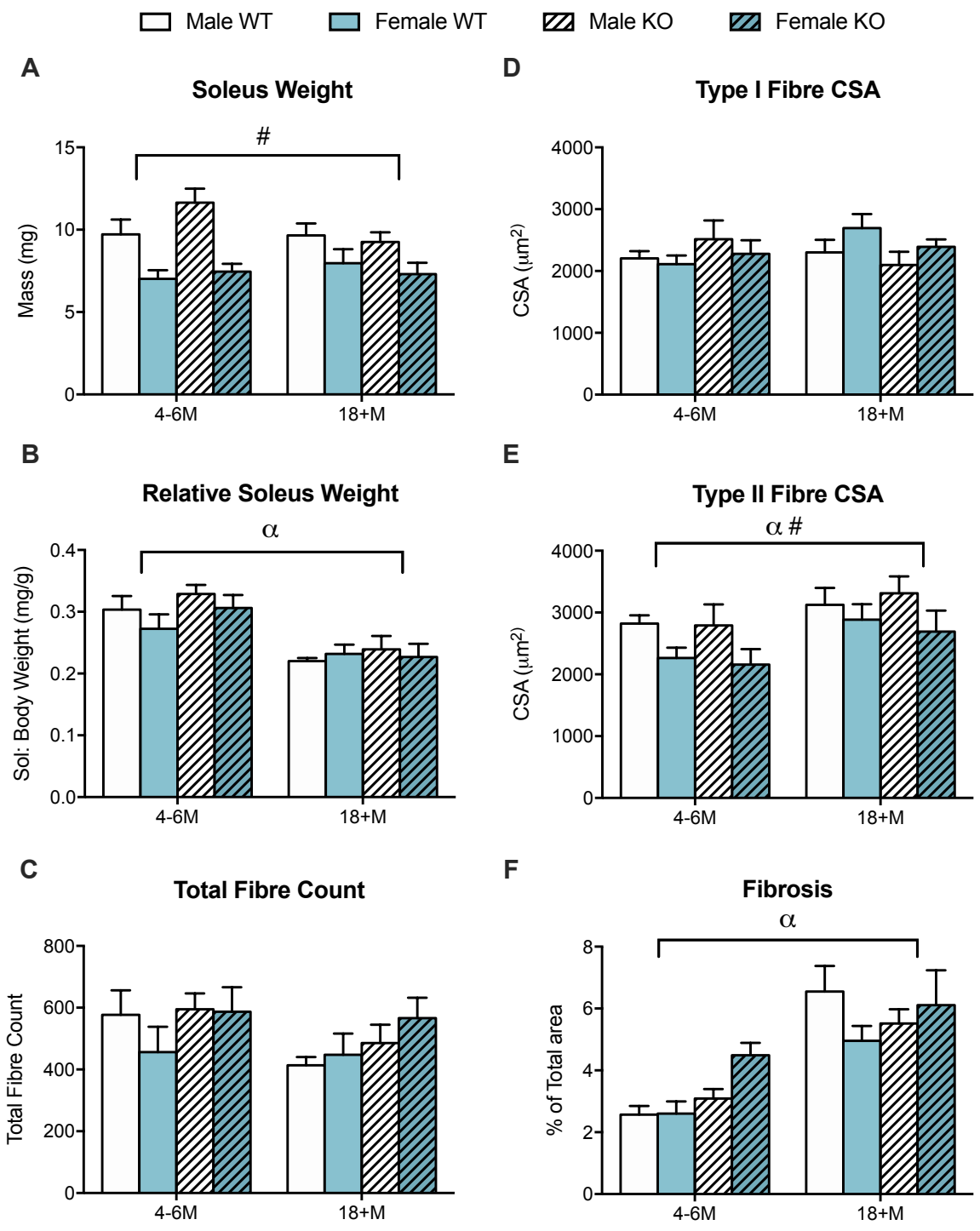


Figure 15. SLN ablation and maintenance of muscle mass with aging

Soleus muscles from adult (4-6M) and older adult (18+M) male and female WT and *Sln*$^{-/-}$ (KO) mice were excised and weighed. Both absolute muscle mass (A) and muscle mass relative to total body weight are displayed (B)(n=7). Total muscle fibre counts, and cross-sectional area (CSA) were examined from fibre typing images and percent fibrosis was determined by Van Geison staining (n=5). α denotes a main effect of age, # main effect of sex, $p \leqq 0.05$. Data are mean ± SEM.

5.4.5 Muscle Function

In addition to muscle fibre type and muscle size, the contractile function was measured on whole soleus muscles from young adult and older adult, male and female, WT and *Sln*$^{-/-}$ mice. Here it was found that age significantly affected absolute twitch force production (Fig. 16A, $p < 0.001$). Changes in absolute twitch force were primarily witnessed in male mice (age x sex $p = 0.059$), as older adult males, regardless of genotype, displayed significant reductions in absolute twitch force compared to young adult, genotype matched males (WT: 38.89 ± 9.72 vs. 22.30 ± 8.93, $p = 0.001$, *Sln*$^{-/-}$: 29.66 ± 3.74 vs. 16.91 ± 5.52, $p = 0.016$) while no differences were found in female groups (WT: 27.02 ± 4.97 vs. 23.96 ± 8.78, $p = 0.517$, *Sln*$^{-/-}$: 29.66 ± 3.74 vs. 22.55 ± 8.63, $p = 0.138$). For absolute maximal tetanic force both age ($p = 0.020$) and sex ($p = 0.005$) were significant factors, with males and young adult animals generally displaying greater maximal muscle force production (Fig. 16B). Both twitch and tetanic measures were also separately normalized to muscle cross-sectional area to examine specific muscle force production. Age persisted as a significant factor in twitch force production when normalized for size (Fig. 16C, $p < 0.001$), with older mice displaying reduced twitch force compared to young adult counterparts. A sex by genotype interaction was also found to be significant ($p = 0.039$), with SLN ablation resulting in significantly reduced specific twitch force in young adult males (50.27 ± 8.71 vs. 33.08 ± 9.87, $p = 0.012$) but not in females (42.74 ± 5.71 46.74 ± 9.24, $p = 0.571$). When examining normalized maximal tetanic force production (Fig. 16D), age remained a significant factor ($p < 0.001$) while sex was no longer significant ($p = 0.567$). Finally, both relaxation (Fig. 16E) and contraction (Fig. 16F) were examined. With respect to contraction rate, sex ($p = 0.339$) and genotype ($p = 0.366$)

were not found to be significant factors while aging was ($p = 0.006$), with older animals generally displaying slower contraction rates. Conversely, for relaxation rate, while age was not significant ($p = 0.187$), genotype significantly impacted relaxation rate ($p = 0.049$), with *Sln*$^{-/-}$ mice generally displaying slower relaxation rates than WT. Similarly, sex was a significant factor ($p < 0.001$) with males displaying faster relaxation rates than female counterparts.

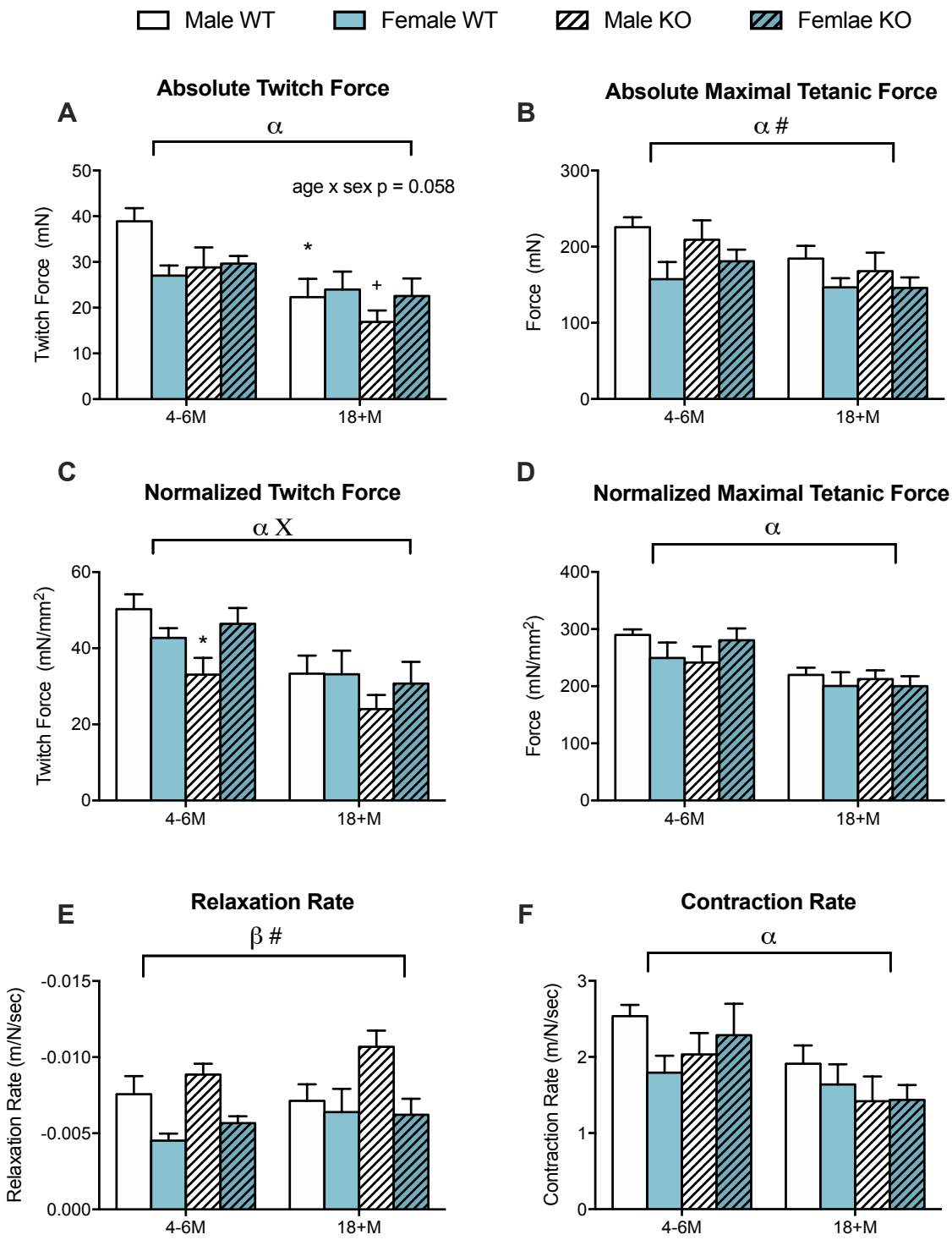


Figure 16. SLN ablation and soleus muscle function in male and female mice with aging.

Muscle function was assessed on soleus muscles of adult (4-6M) and older adult (18+M) male and female WT and *Sln*$^{-/-}$ (KO) mice. Maximal twitch and 100Hz tetanic force expressed in absolute (A & B) and normalized to cross sectional area (C & D). Rates of relaxation (E) and contraction (F) expressed as the maximal rate of force gain or loss. α denotes a main effect of age, # denotes a main effect of sex, ß denotes main effect of genotype, X denotes a genotype by sex interaction, * denotes difference from 4-6M male WT, + denotes difference from 4-6M male KO, $p \leq 0.05$. Data are mean ± SEM (n=5).

5.5 Discussion

This study sought to discern how changes to SLN content impact the activation of Cn signalling and the downstream impact on muscle mass and function in both male and female mice with aging. Aligning with previous work by Lin et al (2018) (163) and Qaisar et al (2019) (160), this study found that SLN expression is increased in skeletal muscle with aging. With the exception of the previous chapters of this thesis, previous research examining SLN expression has been confined to males. This study found females displayed greater SLN expression than males regardless of age and confirmed that, like males, females display an increase in SLN content with aging. As the relative ratio of SLN to SERCA expression impacts cellular Ca^{2+} homeostasis and muscle function, this study also examined SERCA1a and SERCA2a content. With respect to SERCA2a, aging resulted in greater SERCA2a content, predominantly in male animals. Conversely, neither sex or age of the animal impacted SERCA1a expression. As SLN preferentially binds SERCA1a, the increase in SLN content with no change to SERCA1a content with aging witnessed here would suggest greater inhibition of SERCA. This aligns with previous literature showing reduced SERCA function, delayed relaxation rates and increases to basal $[Ca^{2+}]_{cyt}$ in aging muscle (138, 145, 242).

Interestingly, while it is established that Cn plays a pivotal role in signalling for adaptations to muscle fibre type and mass, both of which are impacted by aging, the activation status of the Cn-NFAT pathway has yet to be examined in aging skeletal muscle. As such, this study is the first to report that while aging does not impact the total content of Cn, it does result in a reduction in the ratio of phosphorylated, inactive, NFAT to total NFAT content, suggesting greater Cn activity with aging. This corresponds with previous reports that find Cn is activated

in response to sustained sub-maximal increases in $[Ca^{2+}]_{cyt}$ (249, 250) and the previous studies reporting increases to resting $[Ca^{2+}]_{cyt}$ in aging skeletal muscle (138, 145, 242).

In this study it was hypothesized that SLN expression may be upregulated in aging in order to promote these submaximal increases in $[Ca^{2+}]_{cyt}$ and activate hypertrophic Cn signalling. This particular phenomenon is witnessed in both muscle unloading and muscle disease where SLN is dynamically upregulated and studies examining the ablation of SLN in these models find reduced Cn signalling, reductions in muscle mass and worsened disease characteristics (66, 98, 101). In order to determine whether SLN expression similarly impacts Cn signalling with aging, this study examined Cn expression and the ratio of phosphorylated NFATc1 to total NFATc1 as a measure of Cn activity in both WT and *Sln*$^{-/-}$ mice with aging. Contrary to the hypothesis, SLN ablation had no impact on Cn content and while Cn activation was increased with aging, there were no differences in Cn activity between genotype groups. Additionally, although females were found to display greater SLN content than males in both young adult and older adult animals, there were no sex differences in Cn content or activity, further suggesting that SLN was not required to activate Cn with aging. The data assessing measures of muscle mass and muscle function align with the findings that SLN did not promote Cn signalling with aging. With respect to muscle mass, this study found no differences in soleus muscle weight, total fibre count or the cross-sectional area of type I or type II fibres between WT and *Sln*$^{-/-}$ animals. Similarly, it was hypothesized that the promotion of muscle mass through SLN-Cn signalling with aging would ultimately result in greater muscle function in aged WT animals compared with their *Sln*$^{-/-}$ counterparts, yet the ablation of SLN did not impact maximal tetanic force in older adult animals. Collectively, the Cn activation, muscle

mass and muscle function data in this study suggest that while SLN content and Cn signalling are greater with aging, SLN is not required to activate beneficial Cn signalling as shown to be the case in muscle disease and unloading.

The disparity between this study and the studies reporting that upregulated SLN promotes Cn signalling may be due in part to differences in the extent of SLN upregulation. In this study, aging resulted in a 2.6-fold increase in males and 1.7-fold increase in females, which is much lower than the previous studies examining the relationship between SLN and Cn signalling. In fact, the study by Fajardo et al (2017) reporting that upregulated SLN promoted Cn activity and greater muscle mass with mechanical unloading found a 14-fold increase in SLN content in tenotomised soleus muscles (66). Similarly, the studies examining SLN upregulation in the soleus muscles of various disease models have also shown greater upregulation, including a 9-fold increase in a model of centronuclear myopathy (98) and between 10-20-fold increases in models of DMD (108). Compared to the drastic increase in SLN content in these models, it is possible that a doubling of SLN content with aging is not great enough to activate Cn. Moreover, as SLN ablation did not impact Cn activity in this study, it is yet to be discerned if the age-induced activation of Cn is mechanistically linked to changes in muscle mass. This is specifically relevant as a study by Dunn et al (2000) found that skeletal muscles which overexpress a constituently activated form of Cn (CnA*) were indistinguishable from WT and that Cn activation only resulted in beneficial hypertrophic effects when an additional stimulus, such as muscle overload, was applied (93). This underscores the complexity of Cn-hypertrophy signalling and future studies should examine direct inhibition of Cn to discern its role in the maintenance of muscle mass with aging.

Interestingly, while SLN ablation was not shown to impact Cn signalling, muscle mass, or maximal muscle force production, genotype differences in muscle fibre type were observed. Specifically, there was a main effect of genotype with *Sln*$^{-/-}$ animals generally displaying greater type I fibre counts than WT counterparts, which was the opposite of our hypothesis. Conversely, SLN ablation did not impact the total fibre count or relative proportion of type II fibres. Previous research examining the same *Sln*$^{-/-}$ mouse line found no differences in muscle fibre type between WT and *Sln*$^{-/-}$ young adult males, which was also found in this study. Interestingly, the groups which displayed greater SLN expression, older adult males and both female age groups, displayed greater variation in type I fibre counts between WT and *Sln*$^{-/-}$ muscles. The data showing SLN does not activate Cn and *Sln*$^{-/-}$ animals display greater type I fibre counts are especially incongruent given the established role of Cn-NFATc1 as the primary promoter in MHCI expression. It is unclear why the ablation of SLN would promote an increase in type I fibre counts and further research should seek both to confirm this finding and to discern the underlying mechanism, specifically focusing on fibre type selection during development, a time in which SLN is drastically upregulated.

Furthermore, the findings around SLN ablation and muscle function in young adults suggest an intriguing dimorphism between sexes. In previous research examining young adult male mice, SLN ablation was shown to improve relaxation rate and to reduce twitch force with no effect on maximal force production (22). In the present study, genotype was also a significant factor in relaxation rate with *Sln*$^{-/-}$ animals generally displaying faster relaxation rates. Although there was not a significant interaction effect between sex and genotype, it is of note that the young adult males showed greater differences between WT and *Sln*$^{-/-}$ (-0.0072

vs -0.0093, p = 0.09) than the young adult females (-0.0058 vs -0.0058, p = 0.95). Similarly, when examining twitch force production there was a significant sex by genotype interaction, wherein SLN ablation resulted in lower twitch force in young adult males, with no effect in females. Collectively, the findings in young adult animals seem to suggest that SLN ablation has a greater impact on muscle function in males than females, which is especially interesting given the greater SLN expression witnessed in females. As this study did not directly examine SERCA function, it is challenging to speculate on whether the interaction between SLN and SERCA and the resulting impact on cytosolic Ca^{2+} transients are different between sexes. Alternatively, the diverging impact of SLN ablation on muscle function between sexes may be due in part to fibre type. From studies examining the expression of SLN in different murine muscle groups (27, 31, 64), it is assumed that SLN is confined to type IIA fibres in mice. Given males expressed more type IIA fibres than females, it is possible that the inhibition of SERCA in a greater absolute number of fibres may summate to impact muscle function more in males than females. In order to test this hypothesis, future studies should examine both the direct interaction between SLN and SERCA and single muscle fibre function in male and female mice.

Finally, there are also areas in which this study is in accordance with the current literature. Specifically, this study aligns with the general consensus that dimorphism in muscle mass, fibre type profiles and muscle function exist due to both sex and aging. When examining the effects of sex, females displayed smaller absolute muscle mass, greater type I fibres, fewer type II fibres and lower absolute force generation than their male counterparts. Likewise, aging resulted in a reduction in the total count of muscle fibres, a lower relative proportion of type II

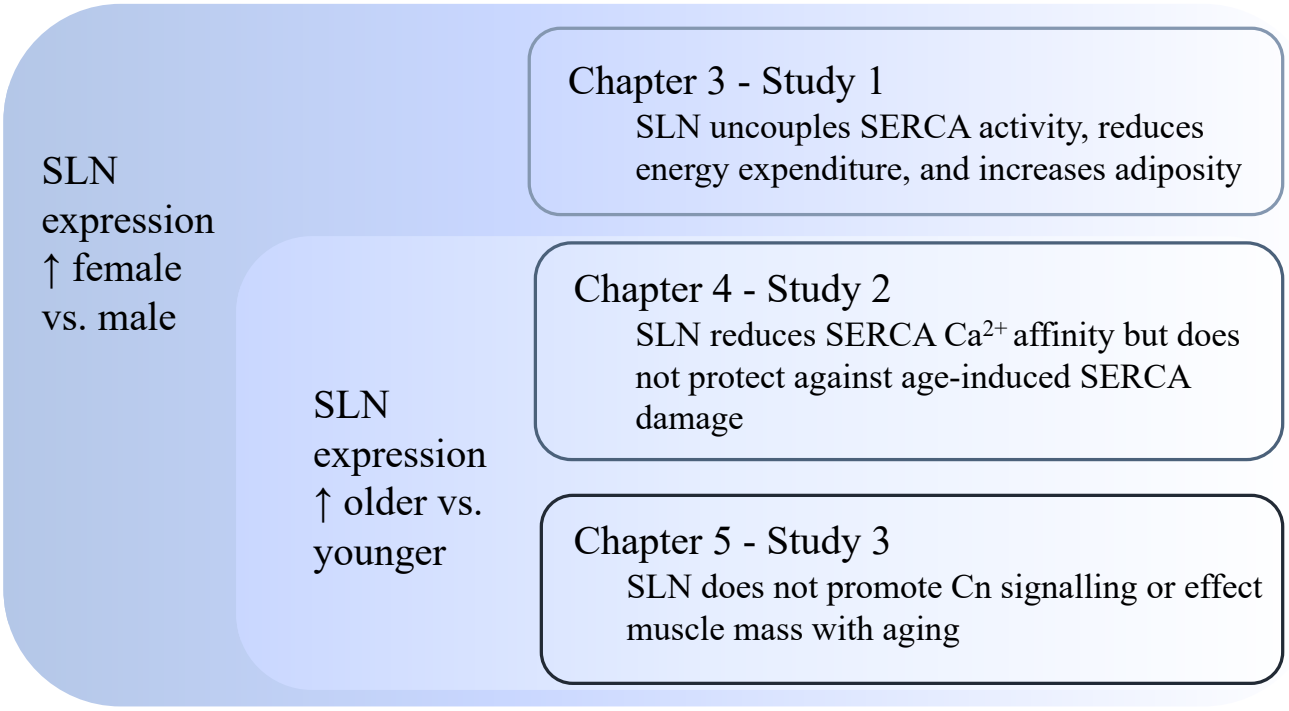
fibres, an increase in muscle fibrosis and lower muscle force generation. In conclusion, this study affirmed many sex and age-related changes to muscle mass, fibre type and function, and presented the novel findings that although SLN does not activate Cn, both SLN content and Cn activity are greater with aging.

Chapter 6: Integrated Discussion and Summary

6.1 Thesis Summary

The aim of the research conducted for this thesis was to examine both sex and age-related changes to the expression of SLN, as well as investigating the role of SLN in promoting energy expenditure, SERCA protection and Cn signalling for muscle maintenance with aging. Notably, the studies of this thesis addressed an important gap in the literature as they were the first to report on SLN expression and function in female mice, with novel sex differences in SLN expression found across all three studies.

Figure 17. Summary of Thesis Findings



Chapter 3 investigated sex differences in SLN expression and the impact of SLN ablation on whole-body energy expenditure in male and female mice. It was found that the soleus muscles of females expressed relatively greater quantities of SLN than that of males, which was accompanied by exacerbated increases to SERCA efficiency and adiposity with SLN ablation in females. In contrast to previous research (48), SLN ablation was found to reduce both absolute and relative whole-body energy expenditure in both male and female mice in this study. However, the ablation of SLN had a greater impact on absolute energy expenditure in males than females, likely due to the greater lean muscle mass of males.

Chapter 4 was the first study to examine sex differences in SLN content with aging, finding that both older age and female sex were associated with greater SLN content. As previous research has shown SLN protects SERCA from heat-induced oxidative damage, this study hypothesized that ablation of SLN in aging muscle would result in greater SERCA damage and reduced SERCA function. While this study added to the body of literature showing greater RONS damage and reduced function of SERCA with aging, it did not provide evidence for the protection of SERCA by SLN in aging muscle. Contrary to the hypothesis, SLN had no impact on SERCA damage and appeared to inhibit SERCA function similarly. Chapter 4 also revealed novel sex dimorphism in RONS damage to specific SERCA isoforms. Interestingly, females displayed greater damage to SERCA1a than males across age groups. As the previous literature examining the susceptibility of various SERCA isoforms to age-related RONS has exclusively examined males, the data from this study may test the current assumption that SERCA2a is more susceptible to age-related RONS.

Finally, Chapter 5 examined the role of SLN in the promotion of muscle mass, fibre type selection and muscle function via activation of Cn signalling in aging mice. Like Chapters 3 & 4, SLN expression was found to be greater in the soleus muscles of both females and older animals. As expected, SLN ablation improved muscle relaxation rates but had no effect on Cn signalling, muscle mass, fibre type or maximal muscle function, suggesting the upregulation of SLN with aging is not recruited to promote beneficial Ca^{2+} signalling, like it is in various unloading and disease models (66, 98, 101). Interestingly, this study found that SLN ablation significantly reduced twitch force in males, a phenomenon previously reported in male mice, with no effect in females. This may be indicative of a sexual dimorphism in the effect of SLN ablation on twitch Ca^{2+} transients and fibre type specific examinations of the impact of SLN on intracellular [Ca^{2+}] may be required to fully understand this finding. Furthermore, although SLN ablation did not impact Cn signalling, this study revealed a novel increase in Cn signalling with age and future studies should investigate the interplay between elevated Cn signalling and the witnessed changes to fibre type with aging in both sexes.

6.2 Sexual dimorphism in SLN expression and impact

Previous research on SLN expression and function have exclusively reported on males, and the studies of this thesis sought to begin eliminating the sex bias in the literature surrounding SLN. One of the most novel and integral findings of this thesis is collective data displaying greater SLN content in the muscles of female mice, which was a consistent finding across all three studies and was true across adult and older adult age groups. In conjunction with greater SLN expression, *Sln*$^{-/-}$ females also displayed lower energy expenditure than WT females, along with acerbated increases to SERCA efficiency and greater adiposity. The data presented

in this thesis should set a precedent for the inclusion of female mice in future studies examining SLN, as the greater SLN expression witnessed in females is more replicative of that witnessed in larger mammals and humans (27).

However, it should be noted that while the expression of SLN was greater in the soleus muscles of female mice, the overall impact of SLN on both absolute whole-body expenditure and muscle function was greater in males. This was witnessed by a greater reduction in absolute whole-body energy expenditure in male *Sln*$^{-/-}$ mice in Chapter 3 and a significant reduction in twitch force with SLN ablation in males in Chapter 5, which was not witnessed between female WT and *Sln*$^{-/-}$ mice. Here, it was speculated that although females displayed greater relative SLN expression within soleus homogenates, the additive effect of relatively less SLN within a greater quantity of muscle mass resulted in larger impacts of SLN on muscle twitch function and absolute energy expenditure in males.

A potential limitation of the studies examining sex differences in the impact of SLN on SERCA function in this thesis, is that data analyses were limited to homogenates in which total protein and fibre type would be mixed. Given the research examining SLN content in various murine muscle groups, it is assumed that SLN expression is exclusive to type IIA fibres, albeit no study has confirmed fibre type specific expression in mice. This expression pattern is especially interesting when considering the implications of greater SLN expression in females with the known sexual dimorphism in fibre type. Previous research, and including Chapter 5, consistently show that males display more type IIA fibres, yet the data from Chapters 3-5 also show greater SLN expression in females, suggesting greater SLN expression in fewer absolute type IIA fibres. It has yet to be discerned if there is a saturation point at which excess SLN no

longer impacts the function of SERCA, and by examining the effects of SLN on SERCA function within homogenates, this study may be altering the ratio of SLN:SERCA ultimately skewing the effect of SLN on SERCA function in vivo. As such there is an opportunity for future studies to examine Ca^{2+} dynamics and muscle function in specific fibre types to confirm the effect of SLN on SERCA function between sexes.

Finally, Chapter 3 sought to lay the foundation for future research on SLN in both sexes, by examining sex differences in SLN expression as well as its impact on SERCA activity and energy expenditure. The novel finding that female muscle displays greater SLN opens a variety of avenues for future research. In addition to examining SLN function in specific fibre types between sexes, further research should examine metabolic differences between WT and *Sln*$^{-/-}$ females. This is imperative work as SLN expression in WT male mice is relatively low, and the greater SLN expression witnessed in WT females would aid in elucidating differences with SLN ablation. Furthermore, this work could uncover a role for SLN in well-established metabolic sex differences, including the promotion of lipid oxidation in females, advancing our understanding of muscle metabolism in both sexes.

6.3 The role of SLN in aging

One of the main objectives of this thesis was to determine if SLN expression in skeletal muscle is altered with aging and to begin discerning the role of SLN expression in aged muscle. In line with previous research (163, 164, 247), Chapters 4 and 5 reported that SLN content in soleus muscles increased with advanced aging, regardless of sex. Research examining muscle disuse, heat stress and disease has suggested that SLN expression is dynamically regulated to protect SERCA function and to promote beneficial Ca^{2+} signalling. Contrary to our hypotheses, SLN

ablation did not result in greater SERCA dysfunction from age-related oxidative stress or activate Cn signalling to preserve muscle mass with aging.

It should be noted that the age range of the older adult mice studied in this thesis may represent a limitation of the studies in Chapters 4 and 5. It has been suggested that 18-24 month C57BL/6J mice are equivalent to 56–69 human years (255); thus, the research conducted here may not be reflective of the expression or impact of SLN with advanced aging. Originally, the older age group in this study was limited to 18-20 months, however adaptations to the research plan in response to Covid-19 lockdowns resulted in the use of older animals with the 18+ age group (ranging from 18-24 months). Here, the 18+ month age group was chosen for several reasons. First, the use of the 18-24 age range for older adult mice is suggested as a best practice by the Jackson Laboratory guide to "Aged C57BL/6J Mice For Research Studies" (256). Studies examining this age group have previously shown significant alterations to cytosolic [Ca^{2+}] and reductions to muscle mass and function which are replicative of human aging (65, 257). Likewise, this study sought to investigate the impact of SLN ablation on SERCA function and muscle mass in healthy older adults, without the contraindications of conditions which may impact mobility and muscle with advanced aging, such as arthritis or age-related tremors. Finally, the age group was chosen in response to the disease and attrition rates witnessed in the particular mouse line used. Specifically, the formation of abdominal tumors was noticeable in this mouse line beginning in many animals around the 16-month mark. This phenomenon did not appear to be due to the genetic ablation of SLN and tumor samples were collected by the animal veterinarian for further examination, with no reports as of the publication of this thesis. Although the age range for the older adult groups was chosen with considerable thought from

both research design and ethical considerations, it may limit the transability of this research to relatively younger older adults. Furthermore, the aging studies of this thesis offer a snapshot of impact of SLN on SERCA protection and Ca^{2+} signalling at one time point in the aging process. The data from this thesis do not provide insights into the temporal changes to SLN expression with aging, thus leaving the potential for having examined an age range which perhaps missed the impact of SLN on aging muscle.

Although the data from this thesis contradicted the original hypotheses, the evidence provided here both adds to the body of literature displaying increases to SLN content with aging and aids in clarifying the role of upregulated SLN in aging muscle. While SLN ablation did not impact SERCA protection or Cn signalling, the examination of SLN ablation and SERCA function with aging in Chapter 4 highlighted that SLN continues to inhibit SERCA function and uncouple SERCA Ca^{2+} uptake from ATPase activity in older adult muscle. Considering that the ablation of SLN resulted in reduced VO_2 in young adult animals in Chapter 3, the increase in SLN expression with aging may have a greater impact on energy expenditure in aged animals. The original intention of Chapter 3 was to examine the effects of SLN ablation on whole-body energy expenditure in both young adult and older adult mice, to discern if the increase in SLN expression in the older animals would amass to greater differences in energy expenditure between older adult $Sln^{-/-}$ and WT animals. However, several animals in the older animal age group experienced extreme stress in the CLAMS cages, with two animals passing away after the 48hr protocol. After consultation with animal technicians, the examination of energy expenditure for Chapter 3 was halted to ensure ethical treatment of the laboratory animals. While this was a very necessary precaution, it leaves a

path for future examination on the effects of SLN ablation on muscle and whole-body energy expenditure with aging that may offer fruitful insights for sarcopenic obesity.

6.4 Conclusion

Sarcolipin (SLN), a small protein inhibitor of SERCA, has been previously shown to alter energy expenditure by reducing SERCA efficiency and to impact both muscle function and cell signalling by altering cytosolic Ca^{2+} transients. The studies of this thesis were the first to examine SLN expression and function in females, uncovering the novel sex differences in SLN expression. Specifically, females displayed greater SLN expression and ablation of SLN resulted in exacerbated increases to SERCA efficiency and adiposity in females. In young adult animals, SLN ablation was associated with greater SERCA efficiency and lower whole-body energy expenditure in both sexes. However, the greater lean body mass of males may amplify the impact of SLN, as SLN ablation resulted in greater reductions in twitch muscle force and absolute energy expenditure in male mice. This thesis also confirmed the findings of previous research, showing that SLN expression increases with age and provides the novel insight that sex differences in SLN expression persist with aging. Here, efforts to discern the role of SLN in aging muscle found that while SLN increased with aging, it did contribute to beneficial Ca^{2+} signalling for the maintenance of muscle mass nor did it offer SERCA protection form age-related oxidative stress. Overall, the data of this thesis have aided in clarifying the role of SLN in aging and uncovered novel sex differences in SLN expression which could have vast implications for muscle metabolism in both healthy and diseased conditions.

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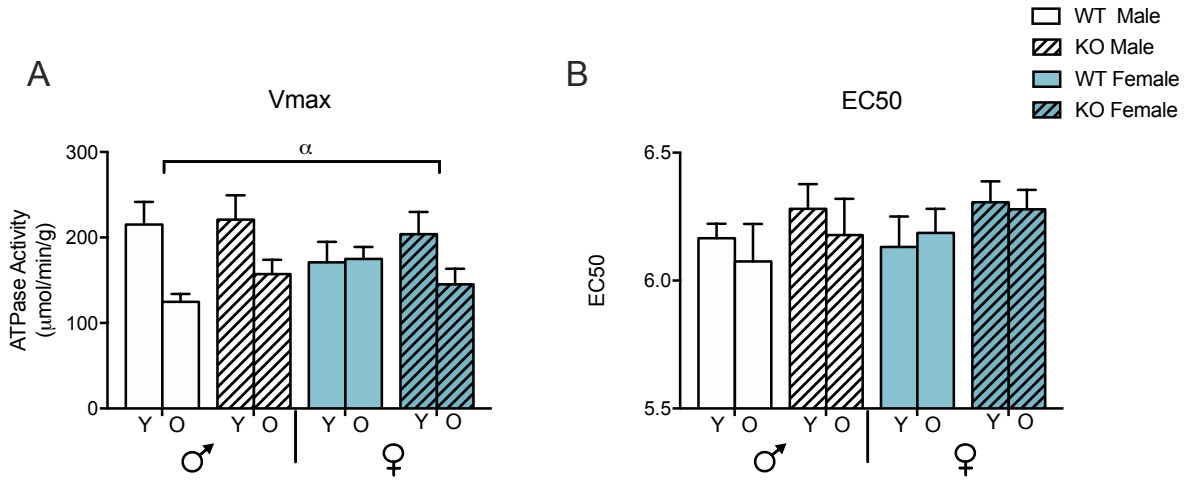
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Appendices

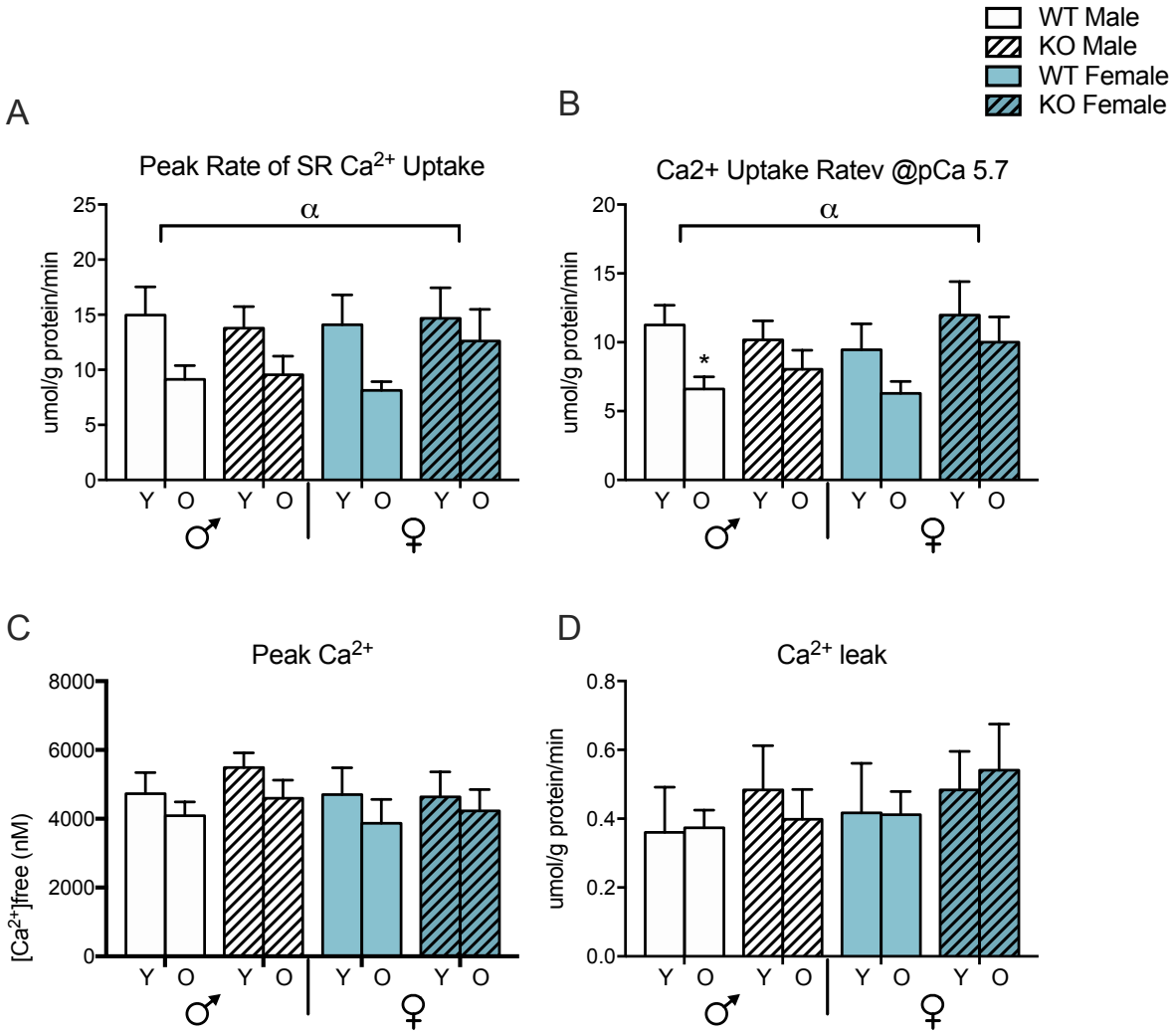
Appendix A – Sex Stratified Data

Sex Stratified Data – Study 2



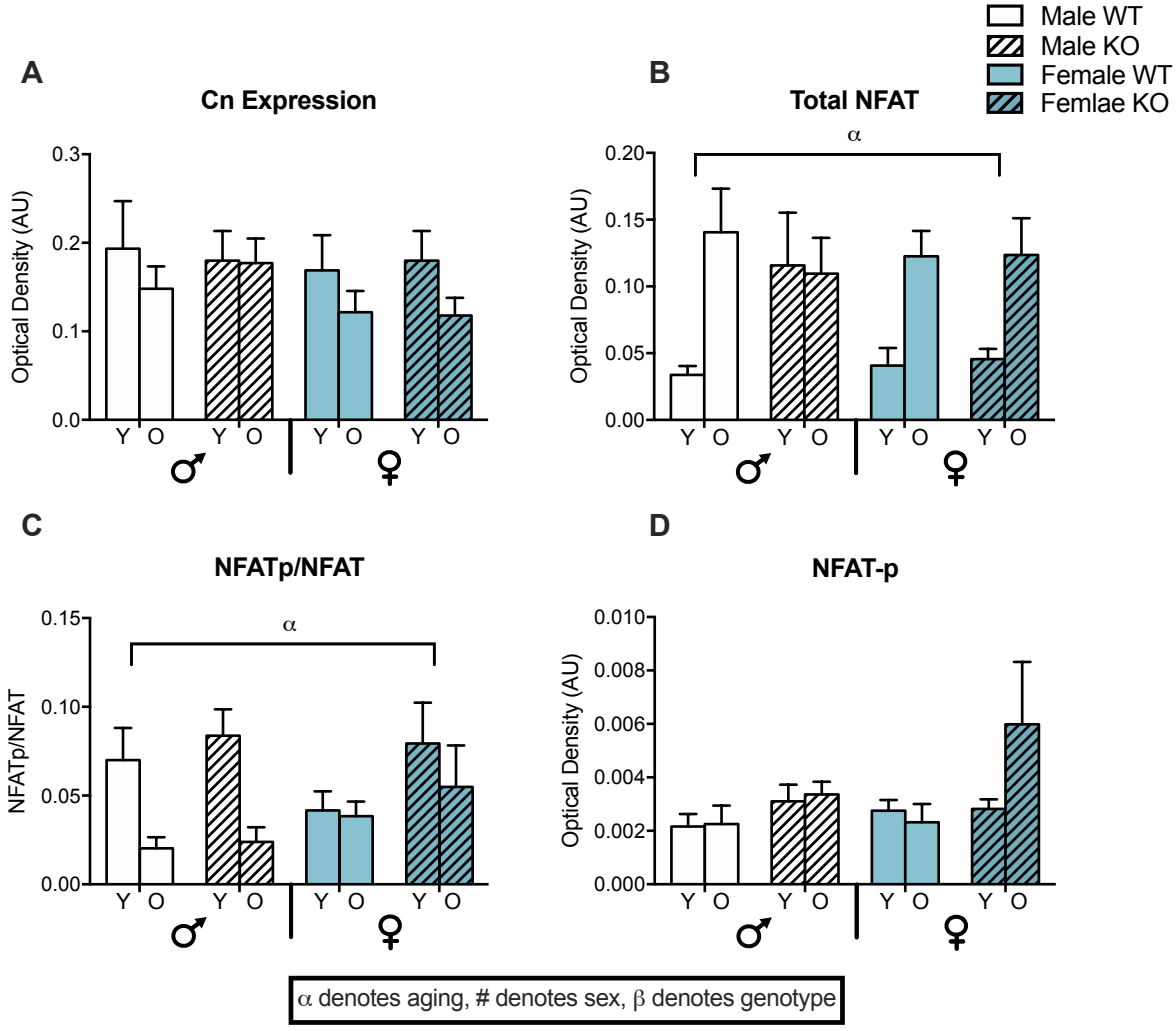
The effect of age, sex and SLN expression on SERCA ATPase activity in soleus muscles.

Ca^{2+}-ATPase activity was analyzed over Ca^{2+} concentrations ranging from *p*Ca 7.6 to *p*Ca 4.8 in soleus muscle homogenates of male and female wild type (WT) and $Sln^{-/-}$ (KO) mice at two age groups: young adult (4-6M) and older adult (18+M). Vmax is the maximal SR Ca^{2+}ATPase activity expressed as µmol per g protein•min^{-1} (A). EC50 is the negative log of the Ca^{2+} concentration required to attain the half-maximal Ca^{2+}-ATPase activity rate (B). α denotes a main effect of age, $p \leqq 0.05$. Data are mean ± SEM (n=5).



The effect of SLN ablation on SERCA Ca^{2+} uptake and leak in soleus muscles with sex and aging. SERCA-mediated Ca^{2+} uptake was assessed in soleus homogenate isolated from male and female young adult (4-6M) and older adult (18+M) WT and *Sln*$^{-/-}$ (KO) mice. Both the peak rate of Ca^{2+} uptake rate (A) was measured in conjunction with the average uptake rate at pCa5.7 (B), both expressed as as µmol•g protein•min. Peak Ca^{2+} was captured as the initial [Ca^{2+}] before Ca^{2+} uptake initiated (C) and SR leak was measured as the rate of change following the addition of the SERCA inhibitor, CPA (D). α Denotes a main effect of age, $p \leq 0.05$. Data are mean ± SEM (n=5).

Sex Stratified Data – Study 3

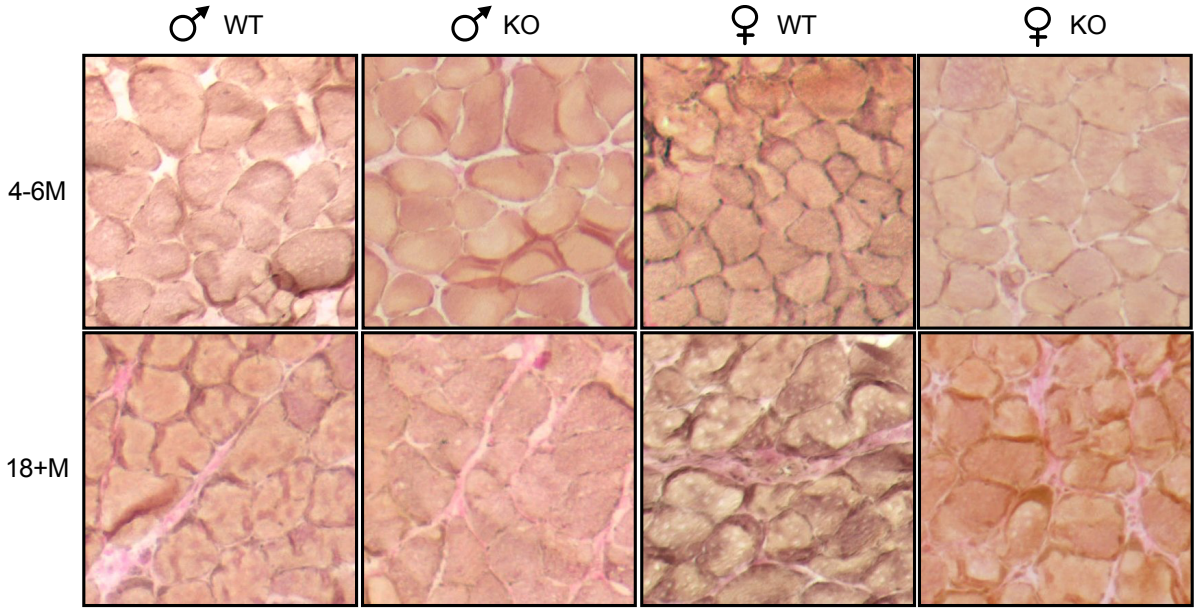


The impact of SLN ablation on Cn signalling with sex and aging

Western blotting for Cn and phosphorylation state of NFAT was conducted on soleus muscle homogenates of male and female, adult (4-6M) and older adult (18+M), WT and *Sln*$^{-/-}$ (KO) mice. Graphed analyses of total Cn (A), NFATc1(B) and NFATc1-p (D) are presented. Optical density for bands of interest were normalized to total protein using Ponceau S staining. The ratio of phosphorylated to total NFATc1 (C) was used to determine Cn activity. α denotes a main effect of age, $p \leqq 0.05$. Data are mean ± SEM (n=6).

Appendix B – Supplemental Data

Representative Images of Fibrosis Staining – Study 3



The Impact of SLN ablation on muscle fibrosis with aging in male and female mice

Van Gieson, staining was conducted on soleus muscle cross-sections from adult (4-6M) and older adult (18+M) male and female WT and *Sln*$^{-/-}$ (KO) mice to assess muscle fibrosis. Representative images of muscle fibrosis presented here, with quantitative analysis in Figure 15.