A cellular memory mechanism in skeletal muscle

A study investigating muscle cell size and myonuclear number after periods of strength training, detraining and retraining

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Abstract

The effects of resistance exercise have shown to be long-lasting. This was previously attributed solely to motor learning, but more recently a memory mechanism residing in the muscle cell itself has been proposed, based on the novel observation that the myonuclei acquired by a former hypertrophic stimulus, remain in the muscle cell during long periods of detraining and may promote subsequent hypertrophy when an additional stimulus is provided.

As such a cellular muscle memory has previously only been found in mice subjected to testosterone treatment, the purpose of the present study was to investigate whether a similar mechanism exists in humans and rats subjected to resistance exercise. The present study utilized a protocol of conventional resistance exercise as training and retraining periods in humans, while training and retraining through housing in a climbing cage was used to resemble strength training in rats. To maximize the hypertrophic response in rats, retraining was also performed by synergist ablation. Changes in muscle fiber size and myonuclear number was examined on isolated single fibers in humans and on cross sections in rats before and after training, detraining and retraining.

In the present study, the human resistance exercise protocol turned out to be unsuitable for testing the cellular muscle memory hypothesis, due to a lack of myonuclear addition during training. The rat climbing protocol, however, caused a significantly higher myonuclear number in trained compared to untrained rats. This elevated number of myonuclei was maintained during detraining, indicating a long-lasting effect. When these muscles were subjected to retraining by synergist ablation, they showed an increased hypertrophic response compared to previously untrained muscles, thus suggesting the existence of a cellular memory mechanism in the skeletal muscles of rats previously subjected to resistance exercise by climbing.

Abbreviations

1RM	1 repetition maximum
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
AMPK	5'adenosine monophosphate activated protein kinase
ATP	Adenosine triphosphate
BW	Body weight
BSA	Bovine serume albumine
CSA	Cross-sectional area
DAPI	4´6-diamidino-2-phenylindole
DEXA	Dual x-ray absorptiometry
EDL	*Extensor digitorum longus*
eIF2B	Eukaryotic initiation factor 2B
eIF3f	The F subunit of eukaryotic translation initiation factor 3
eIF4E	Eukaryotic translation initiation factor 4E
ERK1/2	Extracellular signal-regulated kinase 1 and 2
FAK	Focal adhesion kinase
FHL	*Flexor hallucis longus*
FOXO	Forkhead box containing protein, O-subclass
GIH	The Swedish School of Sport and Health Sciences
GSK3β	Glycogen synthase kinase 3 beta
IGF-1	Insulin-like growth factor 1
MAFbx	Muscle atrophy F-box, also known as Atrogin-1
MAPK	Mitogen-activated protein kinase
MND	Myonuclear domain
MRF	Myogenic regulatory factor

MRF4	Muscle regulatory factor 4
mTOR	Mammalian target of rapamycin
MuRF	Muscle ring finger protein
MyHC	Myosin heavy chain
MyoD	Myogenic differentiation factor
Myf5	Myogenic factor 5
NaOH	Sodium hydroxide
NIH	The Norwegian School of Sport Sciences
p70S6K	70-kDa ribosomal protein S6 kinase
PA	Phosphatidic acid
Pax7	Paired box 7
PBS	Phosphate buffered saline
PCM1	Pericentriolar material 1
PI3K	Phosphatidylinositol 3-kinase
PLD	Phospholipidase D
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel-electrophoresis
TGF-β	Transforming growth factor β
UPS	Ubiquitin proteasome system

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1 Introduction

1.1 Skeletal muscle

Skeletal muscle makes up almost half the body mass in humans and is responsible for all voluntary movement controlled by the somatic nervous system. Skeletal muscle is a heterogeneous tissue containing connective, nervous and vascular tissue as well as muscle tissue, whereas one muscle is comprised of hundreds to thousands of muscle cells, called muscle fibers (MacIntosh et al., 2006). The muscle fibers are controlled by motor neurons, divided into units where one motor unit is a single motor neuron and all the muscle fibers it innervates. When the motor unit is activated, all of its fibers contract (Duchateau and Enoka, 2011). Myofibrils are the smaller compartments of the muscle fiber and are made up of thousands of ordered sarcomeres containing the contractile proteins actin and myosin (MacIntosh et al., 2006) (figure 1.1).

The sliding filament theory proposed by Huxley (Huxley, 1958) is generally accepted as the explanation of skeletal muscle contraction. When a need to exert force arises, an action potential travels down the nerve axon to the neuromuscular junction where acetylcholine is released causing depolarization of the muscle cell. This releases calcium ions from the sarcoplasmic reticulum that binds to troponin, removing tropomyosin from the binding sites of actin. Actin is now available for myosin to bind to, which occurs in sufficient presence of adenosine triphosphate (ATP). The cross-bridge theory (Huxley, 1969) explains how the myosin head with repeated power strokes grabs, pulls and releases the actin filament resulting in shortening of the sarcomeres and contraction of the muscle (figure 1.1).

Motor neurons and their muscle fibers are thought to be recruited in an orderly fashion, always from small to large according to Henneman's size principle (Henneman, 1985; Henneman et al., 1965). This type of orderly recruitment results in a precise control of force and movement (Bawa et al., 2014).

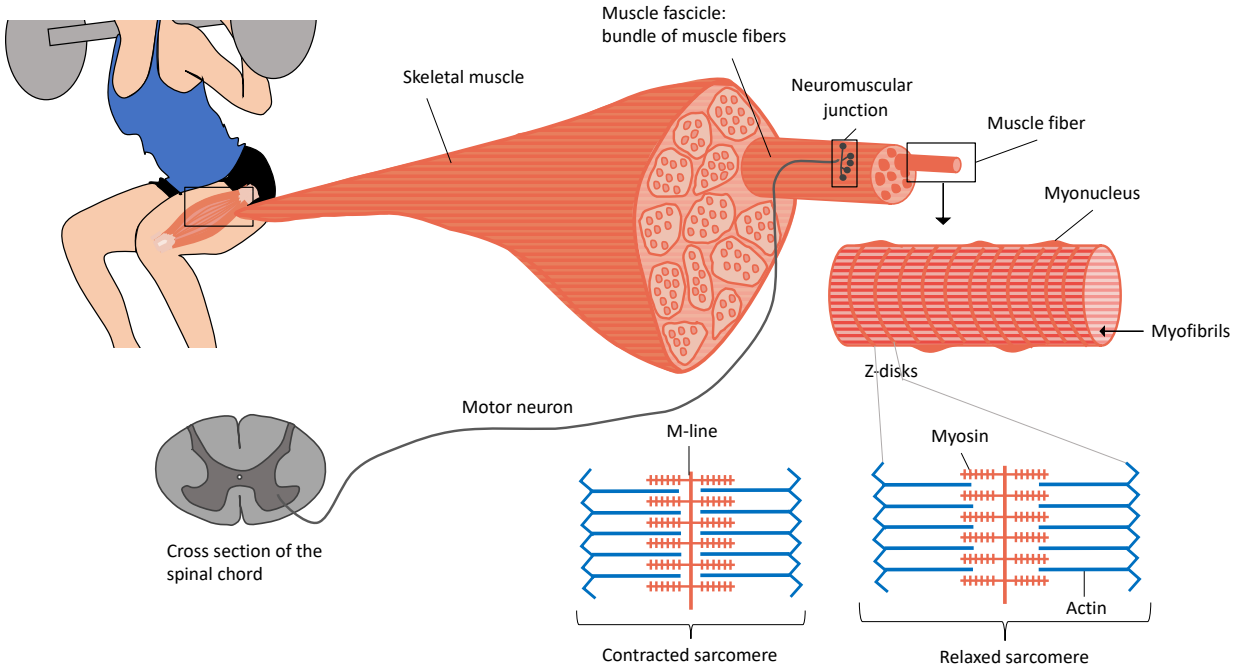


Figure 1.1 The structure of skeletal muscle and the principle of contraction
Skeletal muscles are made up of several muscle fascicles that consists of muscle fibers. Each muscle fiber is comprised of thousands of myofibrils that contain ordered sarcomeres. When an action potential reaches the neuromuscular junction, an increased intracellular concentration of calcium ions causes ultimately the sarcomeres to contract. Contraction occurs when the myosin (pink) grabs and pulls the actin (blue) towards the M-line, shortening the sarcomeres. See main text for details. Figure adapted from Silverthorn and Johnson (2016) and Schoenfeld (2016).

1.1.1 Muscle fiber types

Muscle fibers have differentiated phenotypes, and it is common to group them into different fiber types based on their myosin heavy chain (MyHC) ATPase activity (Schiaffino and Reggiani, 2011). MyHC is found in humans in three different isoforms: type 1 (slow-twitch) type 2A and type 2X (fast-twitch) (Bagley et al., 2017). Muscle fibers can contain a single isoform or a combination of these isoforms, called pure and hybrid fibers respectively. This variation is sometimes referred to as the continuum of muscle fiber types (Galpin et al., 2012).

Previous histochemical and immunohistochemical methods to classify muscle fiber types have shown to misclassify hybrids, hence single muscle fiber type classification through sodium dodecyl sulfate polyacrylamide gel-electrophoresis (SDS PAGE) has been proposed as a more sensitive technique (Murach et al., 2016; Pandorf et al., 2010; Staron et al., 2012). Studies classifying fibers with sensitive single fiber SDS-PAGE have categorized them into seven groups; types 1, 1/2A, 2A, 2A/2X, 2X, 2X/2B, 2B, whereas the last two types only

have been discovered expressed in rodents, even though the corresponding gene is present in humans (Schiaffino and Reggiani, 2011). The different fiber types are distributed variously throughout the body muscles of mammals, dependent on whether the function of the muscle is postural, long-lasting and repetitive, or fast and powerful (Schiaffino and Reggiani, 2011). There has been detected a vast variety in fiber type composition between individuals. For example; the *vastus lateralis* muscle was found to vary from 15-85 % in fiber type 1 composition in a large cohort of humans including both sedentary and active subjects of both genders (Simoneau and Bouchard, 1989). Human studies using single fiber SDS PAGE to characterize muscle fibers, have shown that inactivity causes an increase in hybrids whilst exercise causes specific fiber type changes (Bagley et al., 2017; Borina et al., 2010; Gallagher et al., 2005; Trappe et al., 2007). This indicates that induced by exercise, muscle fibers adapt to specificity. Up to now it is not known to what extent the fiber type composition variability is predominantly caused by genetic variation or training adaptation (Schiaffino and Reggiani, 2011).

1.1.2 Skeletal muscle plasticity

Muscle fiber type transitions are one of many examples of how adaptive skeletal muscle tissue is. Changes in neural activity, loading or hormonal influences can induce a fiber type switch (Schiaffino and Reggiani, 2011). But in response to a variation of stimuli including mechanical loads (resistance exercise), nutrients, cytokines, hormones and growth factors, skeletal muscles can also regulate their fiber size. These stimuli modulate skeletal muscle gene expression and can lead to an increase (hypertrophy) or decrease (atrophy) in the size of preexisting muscle fibers (Egan and Zierath, 2013; Schiaffino et al., 2013). In this thesis, the stimuli that will be mostly focused on is resistance exercise. In order to respond to an increased functional demand during resistance exercise, skeletal muscle can increase its transcriptional and translational capacity by 1) increasing the efficiency of each muscle cell nucleus (myonucleus) or 2) adding new myonuclei through the addition of satellite cells (Gundersen, 2011).

1.1.3 Satellite cells and the addition of myonuclei

Although skeletal muscle has a large degree of plasticity and is able to adapt in order to meet the requirement of the surroundings, skeletal muscle is a post mitotic tissue and does not

undergo significant cell replacement throughout its life. Regeneration of muscle tissue by the aid of satellite cells is therefore crucial for muscle cell repair and remodeling (Montarras et al., 2013). Satellite cells were first discovered by Mauro (Mauro, 1961), and are cells located within the basement membrane of the muscle, separated from the fiber by its own plasma membrane. They are mitotically quiescent but activated when sufficient stimuli, such as muscle damage or mechanical load, is imposed on the muscle fiber. Once activated they progress into the myogenic program becoming highly proliferative and either form myoblasts that multiply and fuse with existing fibers, or they return to quiescence (Verdijk et al., 2014). The former will repair damaged muscle tissue and insert new myonuclei in hypertrophying fibers, while the latter will replenish the satellite cell pool through self-renewal (figure 1.2) (Snijders et al., 2015; Sousa-Victor et al., 2015). Studies have shown that resistance training in humans lead to an increased amount of satellite cells (Herman-Montemayor et al., 2015; Kadi et al., 2004). On the other hand, aging causes a decrease in the satellite cell function (Goodell and Rando, 2015), hence the ability to incorporate new myonuclei and increase the size of the muscle fiber is impaired during senescence (Schultz and Lipton, 1982; Sousa-Victor et al., 2015).

Quiescent satellite cells can be recognized by their expression of the paired box-7 transcription factor (Pax7), and are regulated through the myogenic program by the myogenic regulatory factors (MRFs): myogenin, myogenic differentiation factor (MyoD), myogenic factor 5 (Myf5) and muscle regulatory factor 4 (MRF4) (figure 1.2) (Montarras et al., 2013; Sabourin and Rudnicki, 2000). In addition to the MRFs, insulin-like growth factor 1 (IGF-1) has been implicated to play a role in satellite cell proliferation and differentiation following a bout of resistance exercise (figure 1.2) (Grubb et al., 2014). IGF-1 is often considered the main anabolic hormone that stimulate muscle growth through binding to the IGF-1 receptor activating the later elaborated signaling pathways of P13K/Akt (figure 1.3). In contrast to IGF-1, myostatin, a member of the transforming growth factor (TGF)-β family has been proposed as an inhibitor of hypertrophy (Grobet et al., 2003). Myostatin is a myokine that influences Pax7, myogenin and MyoD, inhibiting satellite cell activation and differentiation (figure 1.2) (Sandri, 2008). Myostatin also activates the Smad signaling pathway that inhibits Akt (figure 1.3) (Fuentes et al., 2013). Notch is another regulator of satellite cell fate as it is proposed to inhibit MyoD and upregulate Pax7 hence promoting self-renewal and inhibiting myogenic differentiation (figure 1.2) (Wen et al., 2012).

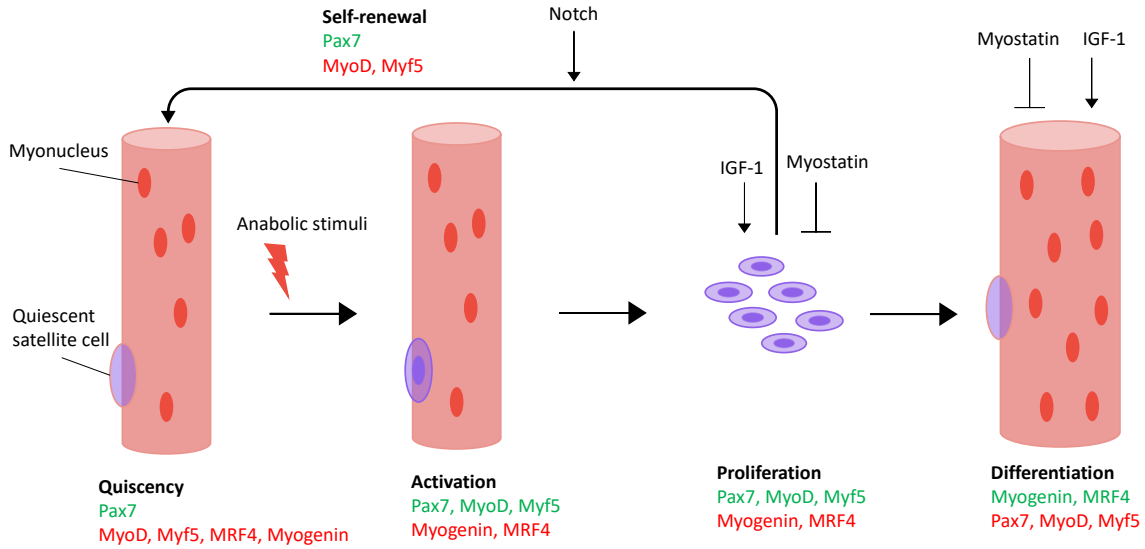


Figure 1.2 Progression of satellite cells through the myogenic program
The satellite cells progression through the myogenic program is determined by the up- and downregulation of the MRFs and Pax7. Proteins marked green are upregulated at the given step of the myogenic program, while proteins marked red are downregulated. See main text for full names and details. Figure based on Bazgir *et al.* (2017) and Snijders *et al.* (2015).

1.2 Hypertrophy

Hypertrophy occurs when the muscle protein synthesis exceeds muscle protein degradation. It is the increase in size of preexisting muscle fibers through the addition of sarcomeres and increase of non-contractile elements. The growth in muscle size is a consequence of an increased cross-sectional area (CSA) in the individual muscle fibers (McGlory and Phillips, 2015). There have also been reported cases where the increased amount of muscle fibers, termed hyperplasia, accounts for an increase in muscle size (Antonio and Gonyea, 1993b). It is, however, generally accepted that hypertrophy is responsible for the main bulk of muscle growth (Adams and Bamman, 2012), as studies showing hyperplasia as the main contributor to muscle growth have been induced using non-physiological conditions in non-mammals (Antonio and Gonyea, 1993a; Antonio and Gonyea, 1993b).

Mechanical tension, metabolic stress and muscle damage are considered the three main mechanisms to achieve muscle growth, at least from a resistance exercise perspective (Schoenfeld, 2010). Mechanically induced tension produced by force generation and stretch, is considered essential to muscle growth and appears to be regulated by the Akt/mTOR pathway (discussed later). Metabolic stress is the accumulation of metabolites as lactate, hydrogen ions, inorganic phosphate and creatine (Schoenfeld, 2013). Several studies support an anabolic role of exercise-induced metabolic stress through the use of blood flow restriction

training (Pearson and Hussain, 2015; Wernbom et al., 2013). Blood flow restriction training takes advantage of the hypertrophic response of metabolic stress and restricts blood flow in order to accumulate metabolites with a lower mechanical load. Thus, blood flow restriction training may be beneficial in circumstances where low load is advisable or preferable due to training periodization, injuries or other. Resistance exercise can result in localized damage to muscle tissue which is theorized to generate a hypertrophic response by the release of various growth factors that regulate satellite cell activity (figure 1.2) (Schoenfeld, 2012). The role of muscle damage in skeletal muscle hypertrophy was recently reviewed (Damas et al., 2018a).

1.2.1 Signaling pathways of hypertrophy

The hypertrophic process of skeletal muscle is regulated by multiple signaling pathways (figure 1.3). In this thesis the focus will be on the pathways considered downstream of mechanical stimuli leading to hypertrophy and disuse leading to atrophy. The phosphatidylinositol 3-kinase (P13K)/Akt pathway is considered to be the master network through which hypertrophy occurs. Akt, also known as protein kinase B (PKB), functions both as an effector of anabolic signals and an inhibitor of catabolic signals (Sandri, 2008). The main downstream effect of Akt signaling is the mammalian target of rapamycin (mTOR) that enhances protein synthesis in several ways (figure 1.3). mTOR promotes transcription of the ribosomal protein S6 kinase (P70S6K) that enhances protein synthesis. mTOR also inhibits the eukaryotic translation initiation factor 4 binding protein (4E-BP1) which is a negative regulator of a mediator of protein translation, the eukaryotic translation initiation factor 4E (eIF4E). In this way mTOR plays an important role in enhancing protein synthesis through inhibiting negative regulators and promoting positive regulators (Miyazaki and Esser, 2009; Schoenfeld, 2010). Akt in addition suppresses the activation of glycogen synthase kinase 3 β (GSK3β) which blocks protein translation initiated by the eukaryotic initiation factor 2B (eIF2B) (McGlory and Phillips, 2015). The P13K/Akt pathway also plays an important role in inhibition of catabolic signals leading to atrophy, as these pathways are tightly regulated (figure 1.3) (Schiaffino et al., 2013). Pathways that regulate the muscle protein synthesis of each myonucleus and modulate satellite cell activity are partly shared, hence figure 1.2 and 1.3 are closely related.

The mitogen-activated protein kinase (MAPK) is a regulator of gene expression, redox status and metabolism, and has been implicated in growth and differentiation of skeletal muscle

(figure 1.3) (Kramer and Goodyear, 2007). The MAPK protein extracellular signal-regulated kinase 1 and 2 (ERK1/2) is upregulated by resistance exercise, and the magnitude of its phosphorylation is dependent on the intensity of the exercise (Kramer and Goodyear, 2007). . An example of how closely related figure 1.2 and 1.3 are, is the work of the previously mentioned important regulator in the activation of satellite cells, IGF-1 (figure 1.2), as it is also known to activate both the MAPK and the P13K/Akt pathway (figure 1.3). It has, however, been shown in both rodent and human studies that mTOR activation can occur independent of growth factors such as IGF-1 (reviewed in Philp et al., 2010), although this mechanism have yet to be fully elucidated. Mechanosensory regulation of protein synthesis has been introduced as an IGF-1-independent mechanism of mTOR activation (Philp et al., 2011). This can occur through high-force contractions during resistance exercise which disrupts the sarcolemma and increases the concentration of membrane phosphatidic acid (PA) through activation of phospholipidase D (PLD) (Egan and Zierath, 2013; Yamada et al., 2012). PA then activates mTOR (O'Neil et al., 2009). Another protein that has exhibited mechanical sensitivity in rodent and human models is the focal adhesion kinase (FAK) that senses load through integrin and activates mTOR (figure 1.3) (Gordon et al., 2001; Philp et al., 2011).

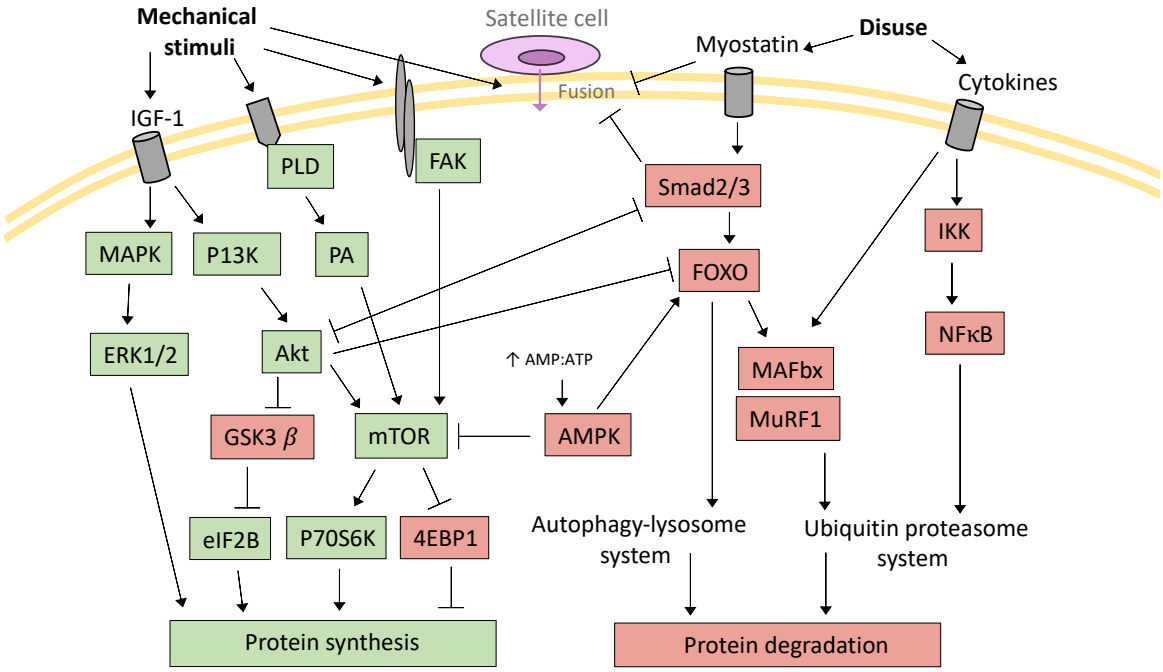


Figure 1.3 Signaling pathways controlling muscle fiber size
This simplified flowchart shows the main signaling pathways associated with muscle hypertrophy (green) and atrophy (red). All the pathways have not yet been fully deciphered. See main text for full names and details. Figure based on Sandri (2008) and Sharples and Stewart (2011).

1.2.2 The role of myonuclei in hypertrophy

Skeletal muscle fibers are the largest cells in the mammalian body with diameters up to 100 μm and lengths in decimeters. In order to support their large cytoplasmic volume, they can contain up to several hundred nuclei, leaving them one of the few cells in the body to be multinucleated (Frontera and Ochala, 2015). Myonuclei are located in the periphery of the muscle fiber between the myofibrils and the basement membrane, and are called myonuclei to be distinguished from the nuclei of other cells in muscle, such as satellite cells, endothelial cells and fibroblasts (Winje et al., 2018).

It has been proposed that each myonucleus control the gene products of a given area of its surrounding cytoplasmic area, termed the myonuclear domain (MND) (Cheek, 1985; Hall and Ralston, 1989). Whether the MND is conserved during hypertrophy has been debated. Studies have shown that the MND is maintained during hypertrophy, as new myonuclei are inserted when the fiber grows (Egner et al., 2016; McCall et al., 1998; Petrella et al., 2008). On the contrary, another study finds hypertrophy to occur without acquiring new myonuclei - causing the MND to increase, as each myonucleus have to control a larger cytoplasmic area (McCarthy et al., 2011). This brings us back to the satellite cell. As satellite cells are the precursor of myonuclei and fuse with myofibers to increase the myonuclear number according to the stimulus, this might be a prerequisite for hypertrophy. When comparing these aforementioned studies, it appeared as hypertrophy up to 15-26 % could occur without the incorporation of new myonuclei (Kadi et al., 2004). Indicating that until a certain limit of myonuclear domain per myonucleus is reached, an increase in fiber area can occur without myonuclear addition. This limit has been referred to as a MND threshold or ceiling (Bazgir et al., 2017; Kadi et al., 2004; Petrella et al., 2006; Van der Meer et al., 2011). Importantly, most studies investigating MND have been done on cross sections of whole muscles or biopsies causing a potential methodological variation due to the thickness of the sections, the imaging technique and the subjective counting of myonuclei. These confounding factors will vary the number of myonuclei.

1.3 Atrophy

Atrophy is the decrease in size of preexisting muscle fibers due to a net loss of proteins, organelles and cytoplasm, occurring when protein degradation exceeds protein synthesis (Sandri, 2013). Atrophy occurs in several conditions such as inactivity, denervation, microgravity or as a result of different diseases (cachexia) or aging (sarcopenia) (Schiaffino et al., 2013). The two major proteolytic pathways that control protein degradation in atrophying muscle are the ubiquitin proteasome pathway (UPS) and the autophagy-lysosome system (figure 1.3) (Milan et al., 2015).

1.3.1 Signaling pathways of atrophy

UPS degrades cytosolic, myofibrillar and nuclear proteins in muscle through the 26S proteasome by ubiquitylation, targeting proteins with ubiquitin (Bilodeau et al., 2016). There has been identified some muscle specific ubiquitin ligase enzymes, that are the enzymes responsible for the addition of ubiquitin to target proteins for degradation. Muscle Atrophy F-box protein/Atrogin-1 (MAFbx) and Muscle RING Finger-containing protein 1 (MuRF1) are the most known ones that target myofibrillar proteins (Bodine and Baehr, 2014). The autophagy-lysosome system refers to the cellular self-degradation process involving selectively engulfment of damaged or dysfunctional proteins, organelles or fractions of cytoplasm by a double-membrane cytoplasmic vesicle called the autophagosome. Subsequently, the autophagosome fuses with the lysosome, degrading and recycling the sequestered cellular components via lysosomal hydrolases (Martin-Rincon et al., 2017; Paquette et al., 2018).

The O-subclass of the Forkhead box containing protein (FOXO) is a family of transcription factors inducing the transcription of genes encoding proteins involved in protein degradation via both the autophagy and the UPS systems, hence function as a major regulator of these two pathways. Overexpression of FOXO3 regulates MuRF1 and MAFbx promoter activities and mRNA expression through potentially different mechanisms (Goodman et al., 2011; Milan et al., 2015). The regulation of FOXO is another example of how tightly regulated the pathways of muscle hypertrophy and atrophy is. FOXO enhances atrophy but is inactivated by Akt-dependent phosphorylation (figure 1.3) (Sandri, 2008).

In a state of cellular stress, such as exercise, AMP-activated protein kinase (AMPK) is activated by an elevated AMP/ATP ratio. Activation of AMPK inhibits energy consuming anabolic processes such as protein synthesis through directly or indirectly affecting mTOR signaling (Goodman et al., 2011). Moreover, activated AMPK stimulates energy producing catabolic processes such as protein degradation through induction of a FOXO3-dependent transcription of MAFbx and MuRF1 (figure 1.3) (Sandri, 2008).

1.3.2 The role of myonuclei in atrophy

While the increase and incorporation of myonuclei during muscle hypertrophy is somewhat substantiated, the myonuclear response during atrophy is far more controversial (Brooks and Myburgh, 2014). In order to maintain the MND, it has been proposed that the myonuclear number increases and decreases along with the growth and shrinkage of muscle fibers. Studies have shown that myonuclei are lost in skeletal muscle of animal and humans during several different atrophic conditions, like disuse-induced atrophy, hindlimb suspension and long term denervation (Allen et al., 1997; Allen et al., 1996; Hikida et al., 1997; Ohira et al., 2002; Rodrigues Ade and Schmalbruch, 1995). However, studies using methods more specifically measuring the myonuclear number have later shown that myonuclei are in fact retained during skeletal muscle atrophy (Bruusgaard et al., 2012; Bruusgaard and Gundersen, 2008; Bruusgaard et al., 2010; Schwartz et al., 2016; Wada et al., 2002). The view of a maintained MND during atrophy has thus been challenged (Gundersen and Bruusgaard, 2008).

The different disuse conditions used in the aforementioned studies to induce atrophy might cause some of the divergent findings. Studies done using non-physiological manipulations such as denervation, immobilization, weightlessness and cancer cachexia, may not give a true picture of what happens under regular physiological circumstances. In addition, due to the localization of the satellite cells, distinguishing them from myonuclei is not easy without specific markers against myonuclei, such as pericentriolar material 1 (PCM1) (Winje, 2018). Studies using solely antibodies against proteins such as dystrophin or laminin to label the circumference of the muscle fiber, thus may be prone to methodological errors such as misinterpretation of true myonuclei, incorporating false positives. Schwartz *et al.* (2016) also report no loss of myonuclei in their study of atrophy in the intersegmental muscles of the tobacco hawkmoth *Manduca sexta*. Not being a vertebrate, the results of this study may be

treated with caution. However, these muscles lack satellite cells and regenerative capacity thereby avoiding the uncertainty of misinterpreting satellite cells as myonuclei (Schwartz et al., 2016).

1.4 Resistance exercise

Resistance exercise is the primary way to obtain muscle growth and can be achieved by a variety of exercise modes; high loads (70-85 % of 1 repetition maximum (1RM)), low loads to failure (30 % 1RM) (Mitchell et al., 2012), low loads with blood flow restriction training (10-30 % 1RM) (Pope et al., 2013), isokinetic training (Lasevicius et al., 2018), body weight training (Counts et al., 2017), and even with different repetition durations as well (Schoenfeld et al., 2015). Comparing different loads, high loads have however been shown superior for increasing strength (Lasevicius et al., 2018). Early-phase adaptations to resistance exercise has been implicated to be predominantly due to neural adaptations (Moritani and deVries, 1979), such as greater motor unit recruitment, synchronization and an increase in maximal rate of force development (Griffin and Cafarelli, 2005). The hypertrophic response has been suggested not to be detected even after several weeks of resistance training (Phillips, 2000). However, this view has been challenged in a recent review where they found muscle growth to occur earlier than previously hypothesized (Counts et al., 2017). Still neural adaptations play a gross role in early-phase adaptations in resistance training, at least for previously untrained subjects (Andersen et al., 2005; Sale, 1988).

1.4.1 Blood flow restriction training

Blood flow restriction training can be used in a variety of different exercise modes (resistance, walking, cycling) and usually involves application of a pressured cuff to the proximal part of each lower or upper extremity. The applied external pressure is sufficient to maintain arterial inflow but restrict venous outflow of blood distal to the occlusion site (Pope et al., 2013). This causes a buildup of metabolites, hence a lot of metabolic stress, but very little mechanical tension as the load is low (10-30 % of 1RM). Blood flow restriction training in combination with low-load resistance exercise have shown not to increase the incidence of muscle damage, neither in young untrained, older or trained individuals (Loenneke et al., 2014; Nielsen et al., 2017; Pearson and Hussain, 2015; Thiebaud et al., 2014; Wernbom et

al., 2013). The hypertrophic response seen in correlation with blood flow restriction training is most likely due to the increased metabolic stress (Teixeira et al., 2018).

1.5 A cellular mechanism of muscle memory

Muscle memory is a term describing previously trained muscles' ability to regain mass and strength faster than previously untrained muscles, even after long periods of inactivity. Often this term is confused with motor learning in the central nervous system (Gundersen, 2016). Motor learning is generally referred to as neuronal changes that allow an organism to accomplish a task better, faster or more accurately than before, after several encounters with the task (Diedrichsen and Kornysheva, 2015). Clearly the aspect of motor learning plays an important role in strength regaining, as the increase in force may precede the increase in mass and therefore may also mask the true hypertrophic response when measured through increases in force output. However, increasing muscle protein is essential for muscle strength, as mass is proportional to power, and solely a neural mechanism for muscle memory has been stated not to be satisfactory (Gundersen, 2016).

Through *in vivo* and *ex vivo* experiments in mice, previous studies in our lab have found a basis to propose a cellular memory mechanism residing in the muscle cell itself (figure 1.4) (Egner et al., 2013). 2 weeks of exposure to testosterone induced larger muscle fibers with more myonuclei than control fibers. These extra myonuclei were not lost 3 months after drug removal, even though the fibers had suffered atrophy and returned to previous size. When these fibers were subjected to overload by synergist ablation - a method to stimulate a hypertrophic response larger than that occurring during resistance exercise - they grew faster than control fibers. Testosterone treatment was used as the initial training period, as it has been proven to cause significant increases in the myonuclear number and the muscle fiber size (Sinha-Hikim et al., 2002; Sinha-Hikim et al., 2003). Testosterone is suggested to promote satellite cell activation, proliferation and differentiation through stimulating Notch and inhibiting myostatin (figure 1.2) (Brown et al., 2009; Kovacheva et al., 2010). Increased IGF-1 signaling, activation of Akt and mTOR, and reduced FOXO3 phosphorylation have also been associated with testosterone treatment (figure 1.3) (White et al., 2013).

As total protein synthesis is the product of the number of myonuclei and synthesis per nucleus, a fiber with a larger myonuclear number provides an increased ability for muscle

protein synthesis, and thus an increased ability to grow (figure 1.4) (Bruusgaard et al., 2010). 3 months in the lifespan of these mice corresponds to approximately a decade in humans, suggesting a long-term memory effect, that is if a similar mechanism exists in humans (Egner et al., 2013). If in fact such a mechanism exists in humans, it may aid in preventing sarcopenia. As the ability to build muscle and recruit new myonuclei is impaired in the elderly (Schultz and Lipton, 1982; Verdijk et al., 2014), there is a potential health benefit in having a larger reservoir of myonuclei to possibly aid in maintaining muscle mass more easily in senescence. Hence strength training in younger individuals would be advisable in order to obtain new myonuclei before this ability declines (Gundersen, 2016).

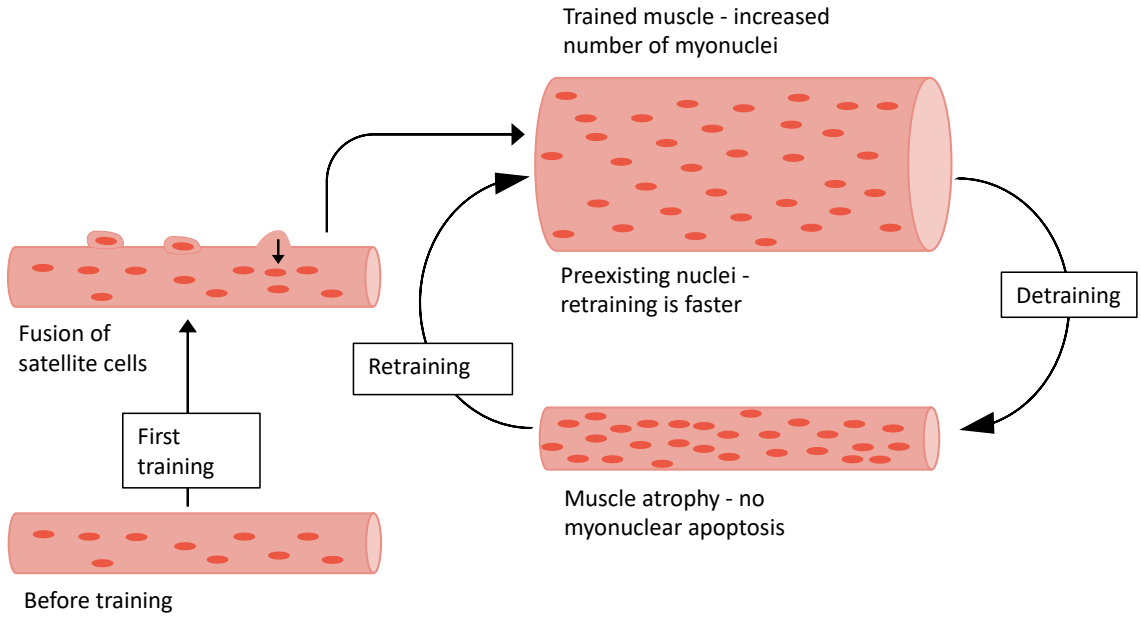


Figure 1.4 The proposed cellular memory mechanism in skeletal muscle
Following the first encounter with resistance exercise a fusion of satellite cells to the muscle fiber occurs, resulting in new myonuclei and muscle fiber growth. As myonuclei are not lost during detraining, the muscle fiber shrinks yet preserves the elevated number of myonuclei induced by training. This elevated number of myonuclei constitute a cellular memory mechanism that promotes regaining of muscle mass during subsequent retraining. Figure adapted from Gundersen (2016).

1.5.1 Previous studies on muscle memory

Apart from the mentioned study on muscle memory, only a handful of other studies investigating the effects of a retraining period has been done. An epigenetic mechanism was proposed recently, in a study that identified previously unstudied genes in muscle hypertrophy that displayed a hypomethylated state and enhanced expression during training. This was maintained during detraining and enhanced by retraining suggesting an epigenetic

muscle memory (Seaborne et al., 2018). Staron *et al.* (1991) strength trained young women for 20 weeks, inducing an increase in CSA. After 32 weeks of detraining, only half of this was reverted and during 6 weeks of retraining, fast muscle fiber types had an increased growth rate compared to the first training period. However, the previously untrained control group experienced similar gains during the retraining period, not support the muscle memory hypothesis (Staron et al., 1991). In a more recent study in older women, an increase in muscle volume was induced after 12 weeks of resistance exercise. After a year of detraining, this increase was completely lost. Retraining for 12 weeks increased the muscle volume once again, but not entirely to the previous volume (Correa et al., 2016). Similar result was found in a study of older men, where 24 weeks of strength training increased the fiber size and detraining for 12 weeks reverted this increase. However, retraining for additional 8 weeks did not induce an increased hypertrophic response compared to the first training period (Taaffe and Marcus, 1997). The same authors published another study in 2009, where older women and men were subjected to resistance exercise for 24 weeks, detrained for 24 weeks and subsequently retrained for 12 weeks. Muscle volume did not increase significantly at any time point (Taaffe et al., 2009). The considerable differences amongst gender, age, measurement methods, training, detraining and retraining durations may be explaining the divergent findings between these studies. All together these conflicting results reveal an additional need for further studies on the human muscle memory. In addition, none of these studies have examined the changes occurring in the number of myonuclei, which is of interest in order to further investigate the proposed cellular memory mechanism in skeletal muscle.

2 Aims of this study

The cellular memory mechanism in skeletal muscle proposed by Egner *et al.* (2013) has still solely been confirmed in mice previously subjected to testosterone treatment and later retrained by synergist ablation. Hence, the aim of this thesis was to examine whether a similar mechanism exists in humans and rats under more physiological conditions. Our aim was to induce a hypertrophic response without the aid of anabolic steroids, but with resistance exercise only. We investigated whether a resistance exercise protocol increased the myonuclear number and the size of muscle fibers. Furthermore, we aimed to answer whether these newly acquired myonuclei remained during a detraining period, and lastly if the excess myonuclei promoted an increased hypertrophic response to retraining.

3 Material and methods

3.1 Human experiments

The human study in this thesis is part of a Human Muscle Memory Project in collaboration with The Swedish School of Sport and Health Sciences (GIH) and The Norwegian School of Sport Sciences (NIH). GIH conducted the resistance training protocol, performed strength tests and sent biopsies to us at the University of Oslo (UiO) for single fiber analysis and to NIH for histochemical analysis on cross sections. As the resistance exercise protocol is of relevance to the results of the single fiber analysis conducted by me, it is included in this thesis.

3.1.1 Subjects and ethical considerations

Twenty healthy but sedentary and previously untrained individuals (N = 10 males, 10 females) aged 24 (± 3) years with height 174 (± 8) cm and weight 71 (± 17) kg were recruited for this study. The participants were interviewed thoroughly to make sure they were suitable for the study. Prior to participation, the subjects were carefully informed of the experimental design, the purpose of the study and the possible risks and discomforts related to the procedures. Participants then signed a written, informed consent. One male subject terminated the training intervention due to personal issues, hence only 9 males completed the study. The study protocol complied with the Helsinki Declaration and was approved by the Regional Ethics Committee of Stockholm, Sweden (DNR 2015/211-31/4).

3.1.2 Resistance exercise protocol

The human resistance exercise protocol lasted for 35 weeks, divided into a 10-week unilateral strength training period, 20 weeks of detraining and lastly a 5-week bilateral retraining period (figure 3.1). During the unilateral training period, the subjects were randomly assigned to train either their left leg (N = 9) or right leg (N = 11) for 10 weeks. In the retraining period, the subjects trained both legs, hence the previously trained leg served as a memory leg, while the previously untrained leg served as a control leg. In both periods, the training intervention consisted of conventional resistance exercise performed 3 times a week, with varying loads

and volume. The exercises used were leg press (Cybex Leg Press, model 16110, USA) and leg extension (Cybex Eagle Leg Extension, model 11051-90, USA). Each week consisted of one session with low load and high volume, one session with the same volume but lower load, and one session with high load and low volume. To maximize hypertrophy, blood flow restriction training was performed during week 4 and week 8 of the unilateral training period. Blood flow restriction training was performed 5 times a week with low load (15-30 % of 1RM). Blood flow was restricted using an inflated cuff regulated to a pressure of ~ 100 mmHg on the proximal thigh during the entire work period. Prior to the study initiation, the participants were adapted to the exercises and equipment through two bouts of familiarization with low loads. Baseline testing was done prior the first week of unilateral training by 1RM tests (Åkerlund, 2016).

Biopsies (2 x 50-100 mg) were taken from the mid-part of *vastus lateralis* under local anesthesia using a Weil-Bladesley's conchotome, as described previously (Henriksson, 1979). Biopsies were taken from the memory leg before and after the training and retraining period, and from the control leg before and after the retraining period. Altogether, 6 biopsies were taken from each participant during the study (figure 3.1). The muscle tissue obtained was rapidly cleaned from blood and fat, fixed in paraformaldehyde (4 %) for later single fiber analysis, or mounted in an embedding medium and frozen in isopentane for later CSA analysis.

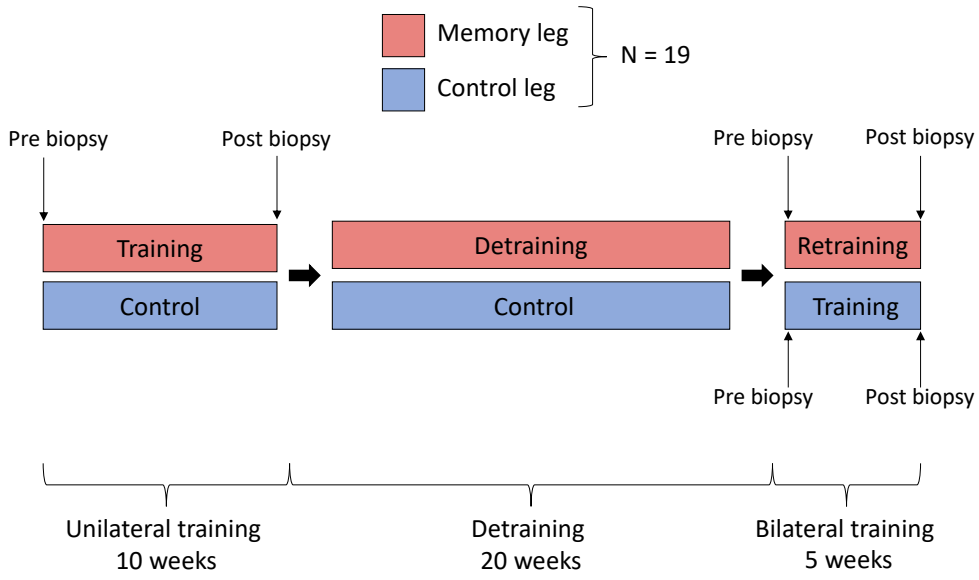


Figure 3.1 Experimental design of the human muscle memory study
The training intervention consisted of 10 weeks of unilateral resistance exercise in the memory (red) leg, 20 weeks of detraining and 5 weeks of bilateral retraining in both memory and control (blue) legs. Biopsies of the *vastus lateralis* were taken before and after training and retraining.

3.1.3 Single fiber maceration

Human single muscle fibers were prepared for analysis by alkali maceration (Wada et al., 2002). All samples were blinded for the analyzer. Biopsy samples were fixed in paraformaldehyde solution (4 %) in phosphate-buffered saline (PBS) (provided from GIH). Fixed samples were dissected into smaller bundles of muscle tissue using tweezers. As much non-muscular tissue as possible was removed before the bundle of muscle fibers was submerged in 0.25 mL 40 % NaOH for 3h at room temperature. 0.75 mL PBS was then added to the solution for neutralization before the dissociating muscle fibers were shaken at 1000 rpm for 8 minutes on a Whirlimixer (Fisons Scientific Whirlimixer) until single fibers were observed. The single muscle fibers were centrifuged at 13 000 rpm (Eppendorf Centrifuge 5417R) for 1 minute. This was repeated 5 times. Between each round liquid was removed without removing muscle fibers and ultrapure water was added up to 1 mL. Isolated single fibers were poured in to a petri dish and water was added until the fibers were completely submerged within it. Single fibers were picked up one by one under a lamp in a dark room and placed on a glass slide (Superfrost Plus, Thermo Fisher Scientific) (figure 3.2). 10-15 fibers were placed on each slide and 5-6 slides were used for each biopsy sample ensuring at least 50 fibers were isolated from each biopsy sample.

The slides were mounted with DAPI Fluoromount-G (Southern Biotech) to stain DNA visualizing the nuclei, as well as mounting the cover slip on the glass slide. 4´,6–diamidino-2-phenylindole (DAPI) is a fluorescent stain that is believed to associate with the minor groove of double-stranded DNA with a preference to adenine-thymine (A-T) clusters (Larsen et al., 1989). As the alkali maceration has been verified to isolate single muscle fibers without non-muscle cells (Wada et al., 2002), all nuclei stained with DAPI were included as myonuclei. Slides were dried overnight and sealed with nail polish in the corner of the slides before image acquiring on a confocal microscope.

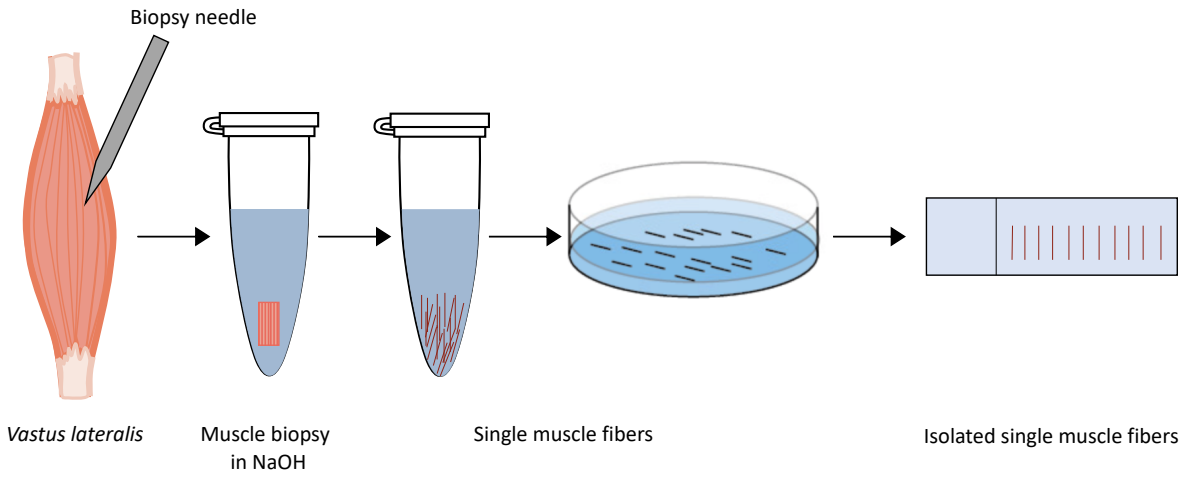
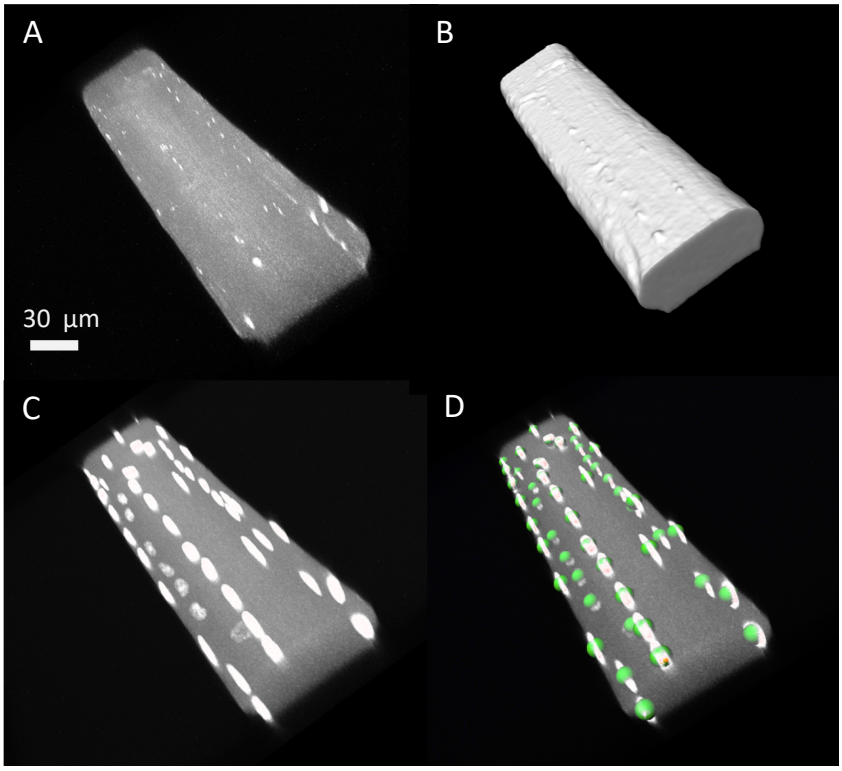


Figure 3.2 Single fiber maceration technique
The muscle biopsy sample is submerged in NaOH (40%) for 3h until single fibers are visual. From a petri dish filled with water, the single fibers are isolated, picked up and placed on a glass slide for single fiber confocal imaging. Described in Wada *et al* (2002).

3.1.4 Imaging and image analysis

Images of human single fibers were visualized on a 40x PlanApo water immersion objective (NA 0.80) on a FluoView FV1000 Olympus BX61W1 upright confocal microscope. A 405 nm laser was used to excite DAPI, visualizing myonuclei. Auto fluorescence from the fiber was illuminated with the 633 nm laser and captured for volume rendering. The transmitted light detector was used to view sarcomeres and general morphology.

Images of the single fibers were acquired (318,08x318,08 µm with an aspect ratio of 640x640 pixels) in different focal planes with a step size of 0,70 µm. A representative image is depicted in figure 3.3. Inclusion criteria for the analyzed fiber segments were; each segment had to be straight, without damage or hypercontraction (figure 3.4 B) and with clearly visual myonuclei (3.3 A, C). The acquired image stacks of the single fibers were reassembled to 3D images using Bitplane Imaris (8.3.1, USA) of which volume was rendered and myonuclear number was counted (figure 3.3 B, D). To correct for potential differences in stretch, the length of 10 sarcomeres were measured in the proximal and distal half of each imaged fiber segment (figure 3.4 A). Fibers with central myonuclei were excluded to avoid including regenerated fibers. 44-57 fibers from each of the 114 biopsy samples were included in the final data material.



Figures 3.3 3D images of a single fiber segment
A) Auto fluorescence of a single fiber segment from the Cy5.5 channel used to render the volume. B) Rendered volume of the single fiber segment. C) Single fiber segment with myonuclei (white dots) stained with DAPI. D) Myonuclei counted automatically (green spheres) and checked manually. Scale bar is 30 µm.

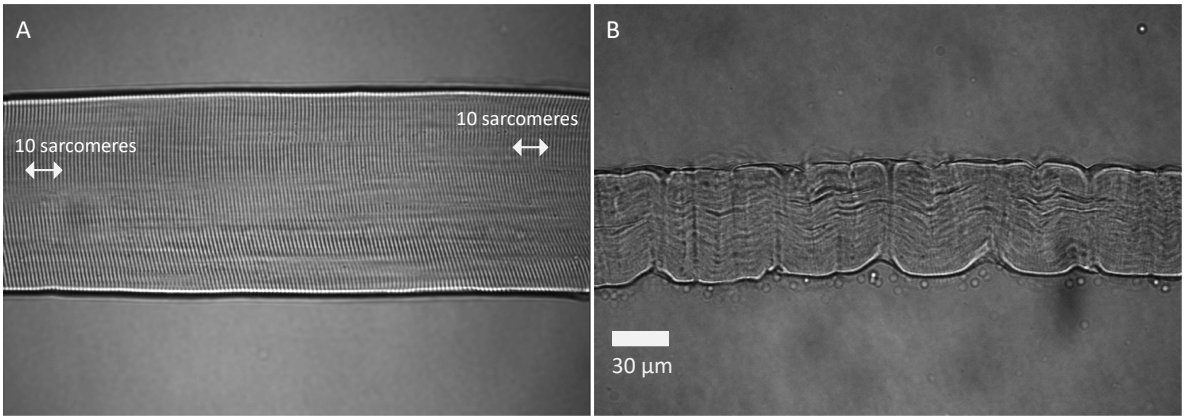


Figure 3.4 Single focal plane images of transmitted light through a fiber segment
A) An intact single fiber segment where the length of 10 sarcomeres at the proximal and distal half of the fiber is measured. B) An example of a hypercontracted fiber, excluded from the analysis. Scale bar is 30 µm.

3.2 Animal experiments

The animal experiments in this thesis are part of a Rat Climbing Cage project conducted at the University of Oslo in the time period of August 2016 to May 2017.

3.2.1 Animals and ethical considerations

Male Sprague Dawley rats, aged 4 weeks at study initiation, were used for the animal experiment. Animals were kept at the animal facility at the Department of Biosciences at the University of Oslo. Rats were housed with a 12 h light/dark cycle with ad libitum access to food and water in the normal cages (detraining period and control rats). For non-terminal experiments the rats were sedated with isoflurane (2 %). For terminal experiments the rats were terminated by cervical dislocation while under deep anesthesia. The animal study was approved by the Norwegian Animal Research Committee before initiation.

3.2.2 Rat exercise protocol

Rats were divided into training and control groups whereas the training group was held in a cage where the rats had to climb to get food (figure 3.5). This served as the initial strength training period and lasted for 5 weeks following a 10-week long detraining period in normal cages. The control group was held in normal cages both during the training and the detraining period. Following the detraining period was a 2-week retraining period, where both the previously trained and previously untrained rats were held in climbing cages. The previously trained group is referred to as the memory group, while the previously untrained group is referred to as the control group. Before the start of retraining by climbing, memory and control rats were divided into retraining and control groups. Rats with similar body weight were put into the retraining group, while rats with dissimilar body weight were put in control group in normal cages. Food intake was measured weekly per cage during training and detraining, and then daily per cage during retraining by climbing in the retrained rats. The right *soleus* muscle of control and memory rats was removed prior to termination after the first training period, after the detraining period and after the retraining period (figure 3.5). The *soleus* muscle was weighed, and the intra-abdominal fat content of the rats was excised and weighed post termination.

In order to maximize the hypertrophic response, unilateral overload by synergist ablation was performed as a retraining protocol in addition to retraining by climbing. Overload of the rats' right *soleus* muscle was induced by tenotomy of the *gastrocnemius*. As *gastrocnemius* is the synergist of *soleus,* tenotomy will induce overload of *soleus*. Synergist ablation was performed unilaterally thus the left leg not undergoing overload served as a contralateral control. In contrast to retraining by climbing, there were no control group not undergoing retraining in this experiment. Hence all rats, independent of their body weight, were retrained by synergist ablation.

At the aforementioned time points (after training, detraining and retraining), the *soleus* muscles were isolated and stored at -80 °C followed by cryo-sectioning. Muscles were embedded in an OCT matrix for frozen sections and sectioned at 10 µm using a cryostat (Leica CM1950). Memory and control sections were placed on the same slide to receive the exact same staining procedure. Sections were stained with antibodies against PCM1, dystrophin and DAPI.

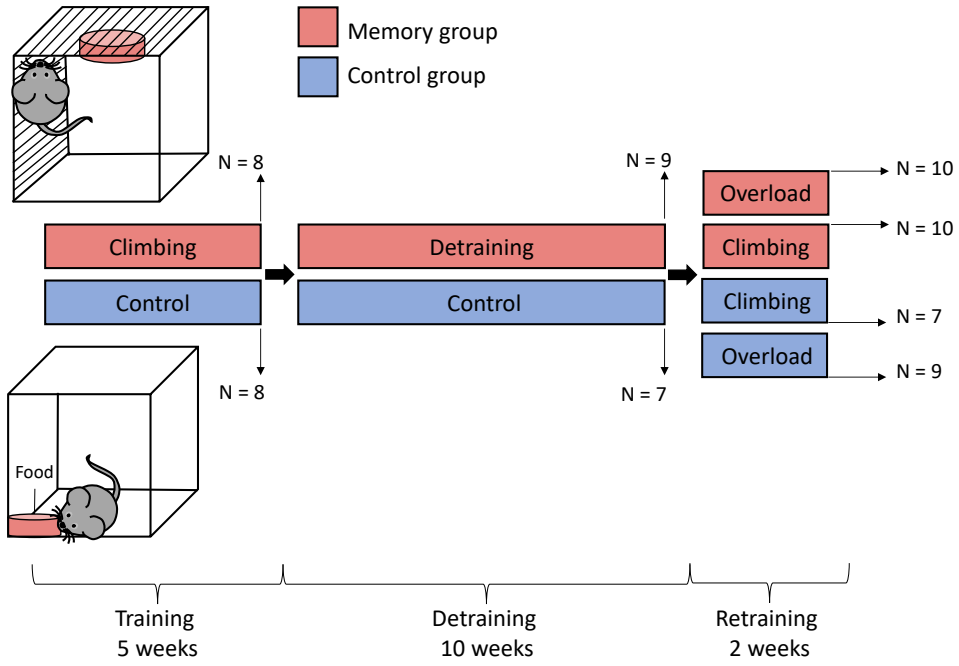


Figure 3.5 Experimental design of the rat muscle memory study
Rats (4-weeks-old) were housed in climbing or normal cages for 5 weeks, making up the first training period. Subsequently, both trained (memory, red) and control (blue) rats were kept in normal cages for 10 weeks of detraining before both groups were retrained for 2 weeks in climbing cages. Additional rats were retrained by synergist ablation for 2 weeks. Rats were taken out of the study at the end of all three periods to isolate the *soleus* muscle for cross-sectional analysis.

3.2.3 Immunohistochemistry

A weakness with previous studies on muscle cross sections has been the interpretation of true myonuclei. By basing the determination of true myonuclei purely on their location in relation to the boundary of the muscle fiber, it may lead to false positives or negatives (Bruusgaard et al., 2012; Bruusgaard et al., 2010). As mentioned in the introduction chapter myonuclei are located in the periphery of the muscle fiber between the myofibrils and the cell membrane. Other nuclei, in particular satellite cells with their close proximity to the muscle fibers, could be misinterpreted as myonuclei and give raise to false positives. Where on the other hand, being too strict on which nuclei to include as myonuclei may lead to false negatives. To overcome this problem, my colleagues have used an antibody against pericentriolar material 1 (PCM1), and verified that it specifically labels myonuclei (Winje et al., 2018). PCM1 is a protein important for microtubule organization and centrosome proteostasis, and is found in the nuclear envelope of adult skeletal muscle (Srsen et al., 2009).

Sections were retrieved from -80 °C and equilibrated for 30 min at room temperature. Prior to staining, sections were pre-incubated with 2 % bovine serume albumine (BSA) in PBS pH 7.4 for 30 min. Sections were stained with a rabbit primary antibody against PCM1 (1:1000, HPA023370, Sigma-Aldrich) in staining solution (5 % BSA in PBS pH 7.4, 0.2 % Igepal CA-630) overnight at 4 °C. Next day the sections were washed 3 x 10 minutes with PBS (1x) and stained with an anti-rabbit secondary antibody (1:1000, AB150077, Abcam, Alexa 488) in 2 % BSA in PBS for 1 hour. Sections were washed 3 x 10 minutes in PBS then stained with a mouse primary antibody against dystrophin (1:20, MANDYS8, 8H11) in staining solution as described above. The sections were again washed 3 x 10 minutes with PBS and stained with a anti-mouse secondary antibody (1:500, A-11005, Thermo Fisher Scientific, Alexa 594) in 2 % BSA in PBS for 1 hour. Sections were washed 3 x 10 minutes with PBS and mounted with DAPI Fluoromount-G.

3.2.4 Imaging and image analysis

Images of the rat *soleus* muscles for analysis was visualized on a 40x PlanApo oil immersion objective (NA 1.3) on a FluoView FV 1000 Olympus inverted confocal microscope, with a resolution of 1024x1024 pixels (317,44x317,44 µm) and dwell time of 2,0 µs/pixel. Laser with excitation wavelength at 408 nm was used to excite the fluorescent stain DAPI to

visualize all nuclei. 488 nm laser was used to excite the green fluorophore of the PCM1 antibody to visualize the myonuclei. Lastly a 594 nm laser was used to excite the red fluorophore of the dystrophin antibody to visualize the border of the muscle fiber, for later fiber size analysis. Image analysis were performed using Adobe Photoshop CS6 (Adobe Systems, USA) and ImageJ (NIH, USA). 6-15 images were acquired from each muscle, 8-23 fibers were analyzed from each image, in total 93-151 fibers per muscle were analyzed. Fibers with their entire dystrophin ring inside of the image were counted, excluding fibers with central nuclei or inconsistent dystrophin ring due to focal plane differences. Nuclei with both PCM1 and DAPI positive staining were counted as myonuclei. CSA was measured at the inside of the dystrophin ring of each included fiber (figure 3.6). The total MND was calculated for each muscle by dividing the total CSA with the total number of myonuclei from all analyzed fibers in that muscle.

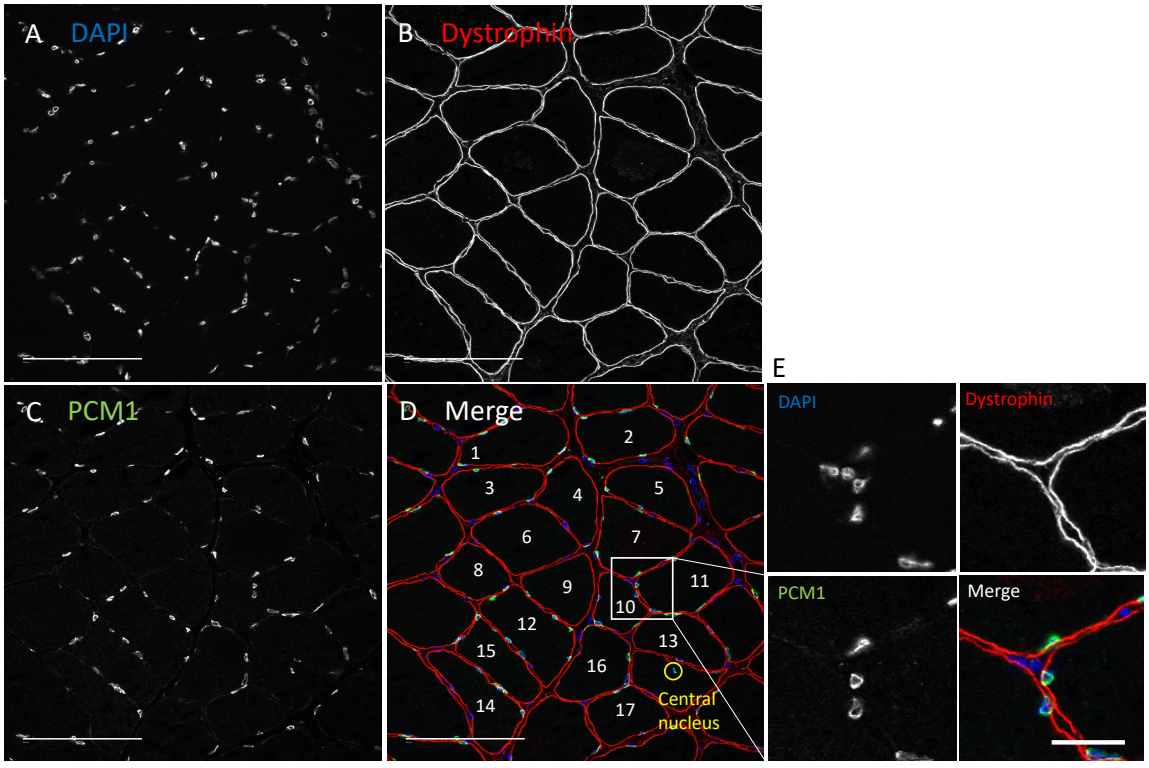


Figure 3.6 Immunohistochemically stained muscle cross section
A) Nuclei stained with DAPI. B) The circumference of the muscle fibers labeled with antibodies against dystrophin. C) Myonuclei labeled with antibodies against PCM1. D) Overlay image of all three channels; DAPI (blue), PCM1 (green) and dystrophin (red), used for cross-sectional analysis. The nucleus marked with a yellow circle represents a central nucleus whereas the entire fiber was excluded from the analysis. All nuclei belonging to the counted fibers marked both PCM1- and DAPI-positive, were accounted as myonuclei. Scale bar is 100 µm (A-D). E) Zoomed in view of the marked square in D. Scale bar is 20 µm.

3.3 Statistics

Statistical analysis was performed with Prism (Version 7, GraphPad) using one way-analysis of variance (ANOVA). For multiple comparisons, Sidak post-test was used, comparing selected groups. In the human study the compared selected groups were the memory leg before and after the first training period, and the memory leg compared to the contralateral control leg before and after the retraining period. The percentage change is shown after correcting against sarcomere length, while raw values are displayed without corrections. In the rat climbing study, the memory group was compared to age-matched controls. For rats retrained by unilateral overload, the overloaded leg was compared to the contralateral control leg. Pearson correlation coefficient was calculated when suitable. The mean of each muscle or muscle biopsy was used as N. All data is represented as mean ± 95 % CI, unless stated otherwise. Statistical significance was set at $\alpha \leq 0{,}05$.

4 Results

4.1 Human muscle memory project

4.1.1 No basis to test the cellular memory hypothesis

Single fiber analysis of the muscle biopsies in the present human study showed no significant increase in myonuclear number after the initial training period (T) (Pre T: 45 ± 6, Post T: 47 ± 6 myonuclei per fiber). Further, no significant changes were seen in the myonuclear number after detraining and retraining in either the previously trained leg (memory) or the control leg (figure 4.1 A-B). There was a non-significant increase in fiber volume (12 %) after training (Pre T :7,4 ± 1,0, Post T: 8,4 ± 1,3 *10^5 μm^3, p = 0,29). After detraining the fiber volume had decreased almost back to baseline (Pre RT: 7,5 ± 1,0 *10^5 μm^3) and did not change during the retraining period, either in the memory or the control leg (figure 4.1 C-D). Since the exercise protocol in this human study did not increase the myonuclear number, this study is considered unsuitable for testing the cellular muscle memory hypothesis.

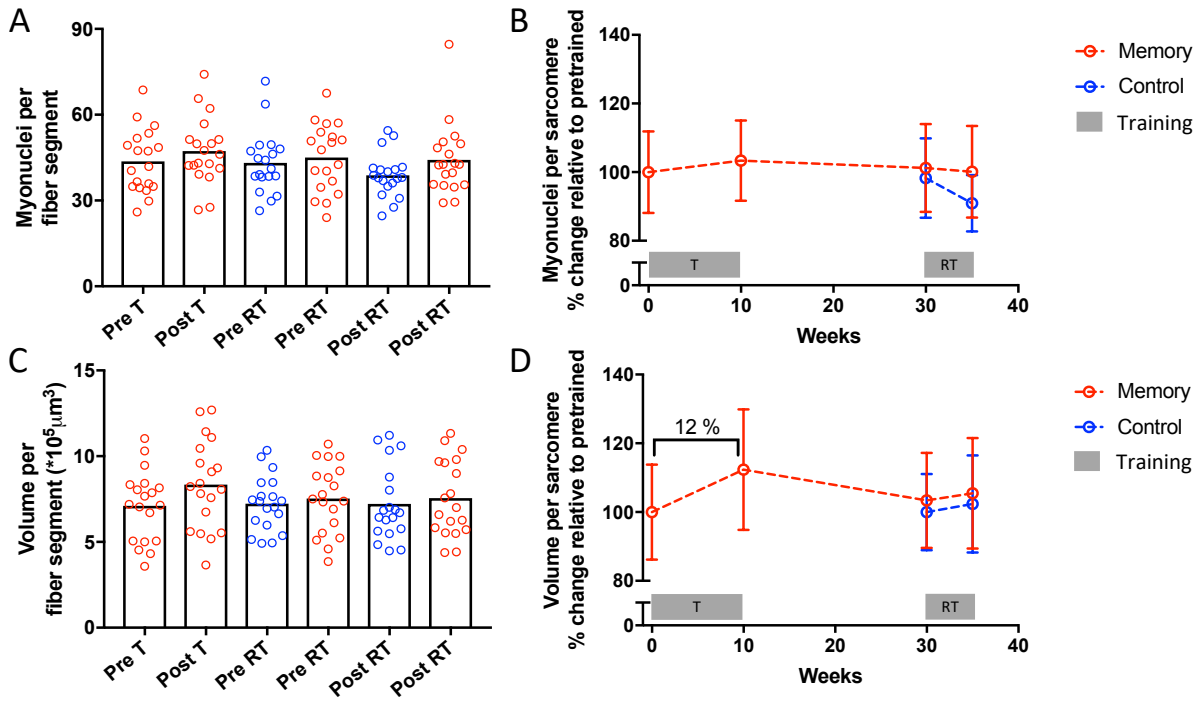


Figure 4.1 No significant changes in myonuclear number and fiber volume during the human study
A) Myonuclei per analyzed fiber segment before and after the training (T) and retraining (RT) period in memory (red) and contralateral control (blue) legs. B) Timeline of percent changes in myonuclear number per sarcomere in memory and control legs relative to their respective first biopsy. C) Volume per analyzed fiber segment before and after T and RT in memory and control legs. D) Timeline of percent changes in volume per sarcomere in memory and control legs relative to their respective first biopsy. Each dot represents a biopsy average (A, C). Data is represented as means ± 95 % CI (B, D). N = 19.

There was a positive linear correlation between the myonuclear number and fiber volume after detraining (figure 4.2). There was, however, no difference between the memory leg ($r^2 = 0,50$) and the control leg ($r^2 = 0,47$). This indicates that the number of myonuclei for a given fiber volume was not increased in the previously trained fibers compared to their controls after training, and therefore there was no basis to test whether the proposed cellular muscle memory exists in humans.

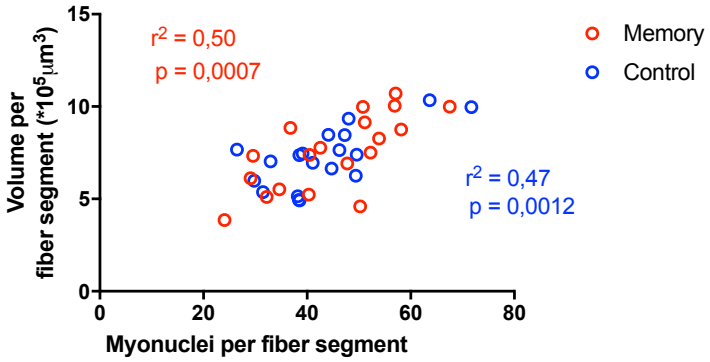


Figure 4.2 Correlation between the myonuclear number and fiber volume after detraining
Volume per fiber segment on myonuclei per fiber segment in memory (red) and control (blue) legs prior to the retraining period. Each dot represents a biopsy average.

4.1.2 Gender-dependent differences and inter-individual responses to training

Neither males nor females had an increased myonuclear number after the initial training period (figure 4.3 A). Males had a non-significantly higher (23 %) fiber volume after the first training period (Pre T: 8,3 ± 1,9, Post T: 10,2 ± 1,5 $*10^5$ μm^3, p = 0,09) while in females it was unchanged (Pre T: 6,6 ± 1,1, Post T: 6,7 ± 1,4 $*10^5$ μm^3) (figure 4.3 B). The inter-individual differences were large in both genders. An example of this inter-individual variation was the myonuclear number before the first training period, which varied with more than twofold in both males (Min: 30, Max: 69 myonuclei per fiber segment) and females (Min: 26, Max: 56 myonuclei per fiber segment) (figure 4.3 A). The inter-individual response also varied. One male subject had a 94 % increase in fiber volume after the first training period, while another decreased by 12 %. In females this variation in response ranged from a 54 % increase to a 48 % decrease in fiber volume (figure 4.3 B).

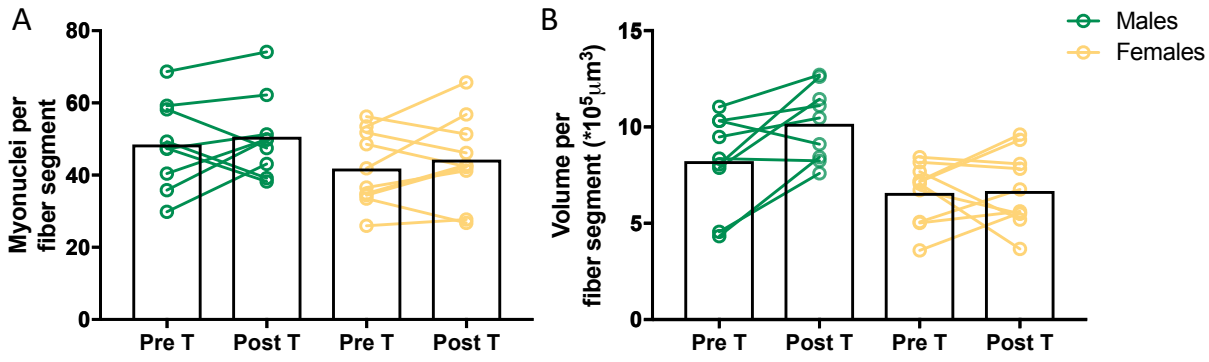


Figure 4.3 Gender-dependent changes in myonuclear number and fiber volume during the initial training period
A) Myonuclear number per fiber segment before and after the initial training period (T), divided into males (green) and females (yellow). B) Volume per fiber segment, before and after T. Each dot and line represent the biopsy average of the same subject before and after T. Bars show group mean.

4.2 Rat muscle memory project

4.2.1 Climbing decreases the food intake, body weight and fat content of trained rats

In the rat climbing study the control rats were kept in normal cages with free access to food for 19 weeks, while the trained rats (memory) had to climb to get food for the first 5 of these weeks. During the 5 weeks of training by climbing, the trained rats had a lower weekly food intake compared to the control group (figure 4.4 A). After the first training period, the memory rats (318 ± 9 g) had a 9 % lower body weight compared to the control rats (348 ± 23 g). This difference was not significant. The memory group maintained a lower body weight compared to the control group during detraining. Retraining by climbing caused a decrease in body weight and food intake in both retraining groups, while the groups not retraining retained both parameters (figure 4.4 A). The previously untrained rats had a lower food intake than previously trained rats during the first days of the retraining period (figure 4.4 B).

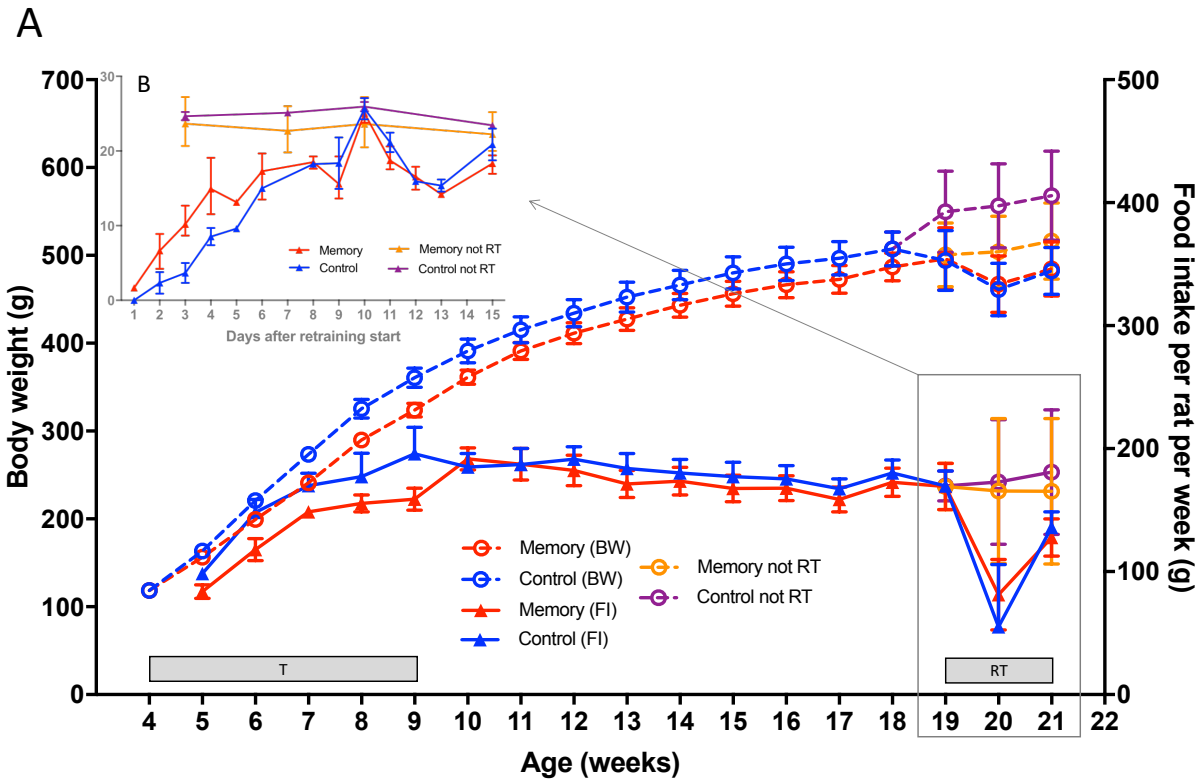


Figure 4.4 Timeline of body weight and food intake of the rats during the climbing study
A) Timeline of the body weight (BW, left y-axis) and mean weekly food intake per rat (FI, right y-axis) in memory (red) and control (blue) groups, and their respective control groups held in normal cages during the retraining period (orange for memory and purple for control). B) Food intake per rat per day from cage average in memory and control rats held in climbing and normal cages during the retraining period. Data is represented as means ± 95 % CI.

After the initial training period, the trained rats (3,0 ± 0,3 g) had 28 % less intra-abdominal fat compared to the control rats (4,1 ± 0,9 g) (figure 4.5 A), but there was no difference in muscle weight between the two groups (figure 4.5 B). 10 weeks of detraining reverted the difference in fat content. At this point the memory group (172 ± 12 mg) had an 8 % lower muscle weight compared to the control group (187 ± 22 mg) (figure 4.5). The memory rats (19 ± 4,4 g) had 10 % less intra-abdominal fat compared to control rats (21 ± 3,8 g) after 2 weeks of retraining by climbing, but there was no difference in muscle weight (figure 4.4 B-C). None of these differences were significant.

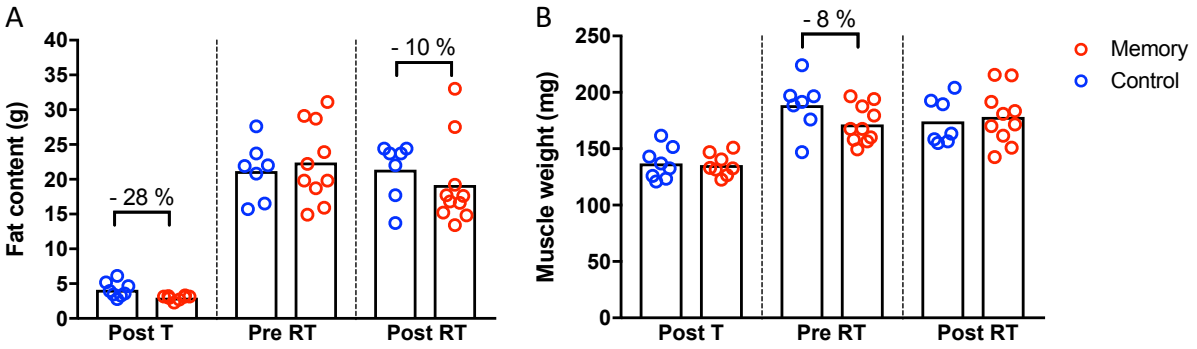


Figure 4.5 Fat content and muscle weight of the rats during the climbing study
A) Intra-abdominal fat content in memory (red) and control (blue) groups after training (T), and before and after retraining (RT). B) Muscle weight of the *soleus* muscle in memory and control groups after T, and before and after RT by climbing. Each dot represents a rat average.

4.2.2 Previously trained rats have a higher myonuclear number

Fiber cross-sectional analysis of the rat *soleus* muscles showed a significantly higher myonuclear number in the trained rats (1,8 ± 0,3 myonuclei per fiber) compared to the control rats (1,3 ± 0,2 myonuclei per fiber) after the initial training period by climbing. The number of myonuclei was still elevated and significantly different in memory rats (3,0 ± 0,2 myonuclei per fiber) compared to age-matched controls after 10 weeks of detraining (2,5 ± 0,2 myonuclei per fiber). Due to normal growth, the myonuclear number and CSA also increased independently of the intervention (Enesco and Puddy, 1964). Hence, the relative difference between the two groups was larger after training (43 %) than after detraining (19 %), even though the absolute difference was similar. In other words, myonuclei were not lost during detraining. After retraining by climbing, the difference in myonuclear number had evened out between the memory (3,3 ± 0,1 myonuclei per fiber) and the control group (3,1 ± 0,3 myonuclei per fiber), only differing with 6 % (figure 4.6 A-B). The trained group (2317 ± 266 μm^2) had a 15 % larger CSA compared to controls (2009 ± 307 μm^2) after the initial training by climbing. This difference was not significant ($p = 0,16$). After detraining, the fiber size increase was completely reverted in the memory group (2908 ± 215 μm^2). Retraining by climbing caused a non-significantly larger CSA (7 %) in previously trained (2833 ± 241 μm^2) compared to previously untrained rats (2637 ± 196 μm^2) (figure 4.6 C-D).

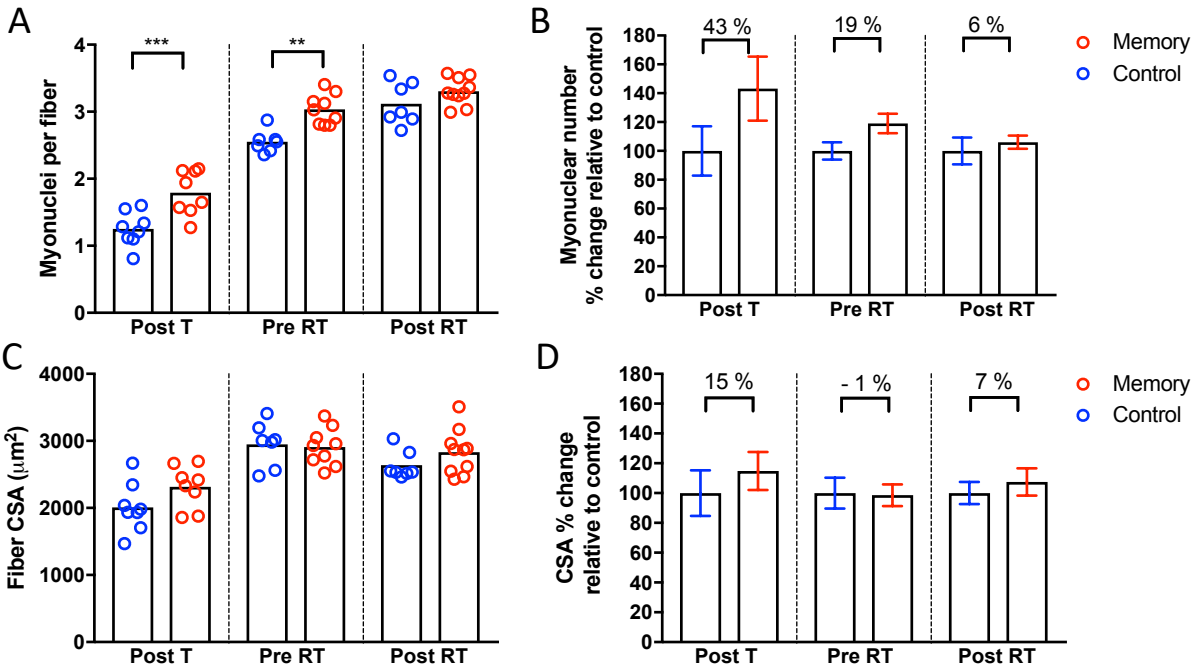


Figure 4.6 Changes in myonuclear number and cross-sectional area (CSA) in the rat climbing study
A) Myonuclear number in memory (red) and control (blue) rats after training (T), and before and after retraining by climbing (RT). **: P = 0,013. ***: P = 0,0003. B) Percent changes in myonuclear number in memory groups relative to their age-matched control group. C) CSA in memory and control groups after T, and before and after RT. D) Percent changes in CSA in memory groups relative to their age-matched control group. Each dot represents a muscle average (A, C). Data is represented as means ± 95 % CI (B, D).

In contrast to figure 4.2 in the human study, the correlation plot between myonuclear number and CSA was different in the memory ($r^2 = 0,10$) and the control group ($r^2 = 0,32$) after detraining (figure 4.7). For a given fiber CSA, the number of myonuclei was larger in previously trained than in previously untrained rats, as figure 4.6 A and C together indicates. Hence our rat climbing model provides a basis for testing our cellular muscle memory hypothesis.

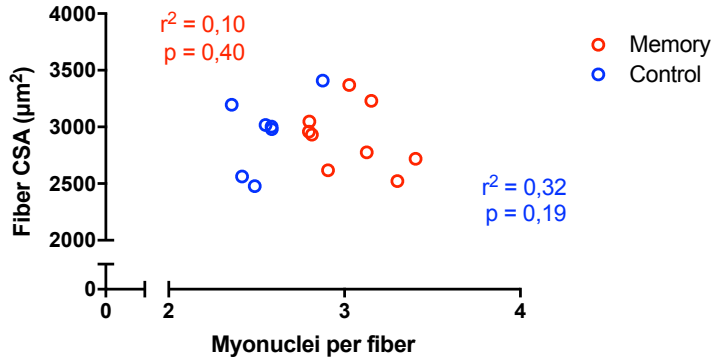


Figure 4.7 Correlation between the myonuclear number and cross-sectional area (CSA) after detraining.
CSA per fiber cross section on myonuclei per fiber cross section in memory (red) and control (blue) muscles after the 10-week detraining period. Each dot represents a muscle average.

4.2.3 Retraining by overload induces a twofold increase in fiber size of previously trained rats compared to controls

Retraining by climbing failed to confirm the cellular muscle memory hypothesis, as the memory group did not grow significantly more than the control group (figure 4.6). In addition to retraining by climbing, retraining by synergist ablation was done to maximize the hypertrophic response, as synergist ablation has previously been shown to induce hypertrophy in 2 weeks (Nakada et al., 2016; Snow, 1990). In this part of the study, memory rats had previously trained in climbing cages for 5 weeks and were after 10 weeks of detraining subjected to unilateral overload by synergist ablation for 2 weeks (OL). The contralateral leg not subjected to overload was used as a control (C).

Retraining by overload caused a significant increase in myonuclear number in the previously untrained rats (C: 2,8 ± 0,2, OL: 3,1 ± 0,2 myonuclei per fiber). The increase in myonuclear number was lower and not significant in the previously trained rats (C: 2,9 ± 0,1, OL: 3,0 ± 0,2 myonuclei per fiber) (figure 4.8 A-B). Both previously trained and previously untrained rats experienced significant gains in CSA during retraining by overload. The memory group increased CSA by 20 % (C: 2427 ± 133, OL: 2919 ± 216 μm^2) while the control group increased by 11 % (C: 2951 ± 148, OL: 3264 ± 297 μm^2) (figure 4.8 C-D), supporting our cellular muscle memory hypothesis.

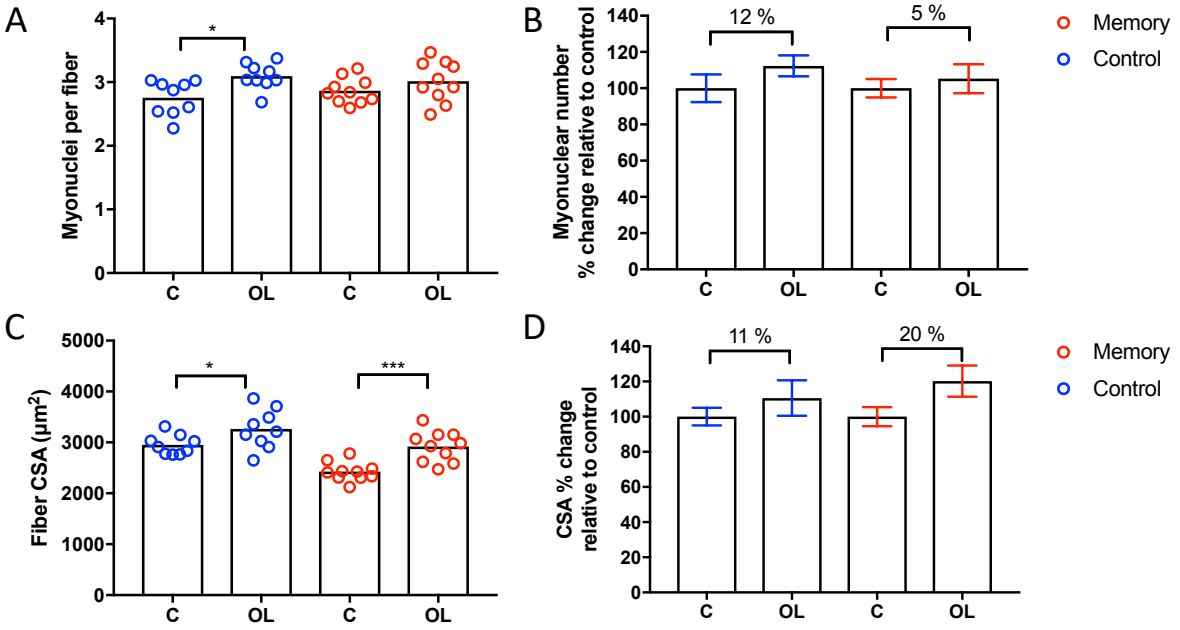


Figure 4.8 Myonuclear number and cross-sectional area (CSA) after retraining by overload
A) Myonuclei per fiber cross section after retraining by overload in memory (red) and control (blue) groups in overloaded leg (OL) and their respective contralateral control legs (C). *: P = 0,024. B) Percent changes in myonuclear number in OL relative to its C. C) CSA per fiber in all groups after retraining by overload. *: P = 0,045, ***: P = 0,003. D) Percent changes in CSA in OL relative to its C. Each dot represents a muscle average (A, C). Means ± 95 % CI (B, D).

When calculating the effect size (cohens *d*) of the two groups during retraining by overload, it suggests that the previously trained rats (1,97) have an increased ability to grow compared to the control rats (1,02), supporting our cellular memory hypothesis (figure 4.9). Cohens *d* is often used to accompany ANOVA and is used to indicate standardized difference. Both the effect sizes were ≥ 0,8, hence considered large, supporting figure 4.8 C. Further advocating figure 4.8 D, cohens *d* shows that the effect of the retraining is almost twice as high in the memory group compared to the control group. So, even though the CSA of the muscle fibers in the control groups started off larger than in the memory group, in relative terms the memory fibers grew twice as much during the 2 weeks of retraining by overload (figure 4.8).

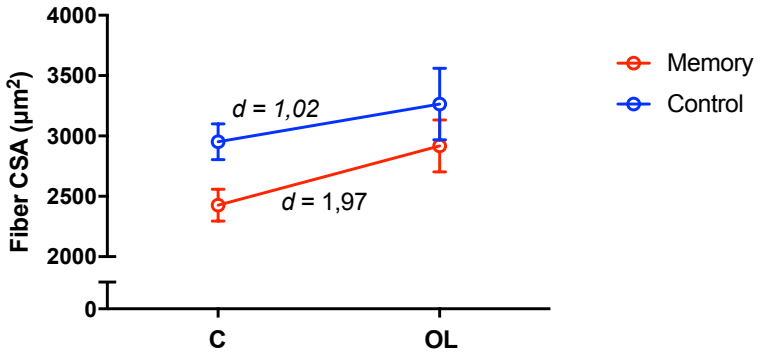


Figure 4.9 Previously trained rats increase their cross-sectional area (CSA) more than control rats
CSA of the memory (red) and control (blue) group after retraining by overload. The line is drawn between the contralateral control leg (C) and the overloaded leg (OL). *d* = cohens *d*. Means ± 95 % CI.

4.2.4 Control rats have a higher bodyweight after retraining by overload

As seen in figure 4.8 C and 4.9 the untrained group started the retraining with a larger CSA than the previously trained group. Terminally after the 2 weeks overload-period, the control rats (627 ± 61 g) had a significantly higher body weight (14 %) compared to the memory rats (551 ± 72 g) (figure 4.10 A). Both the memory group (C: 217 ± 23, OL: 285 ± 26 mg) and the control group (C: 240 ± 18, OL: 291 ± 29 mg) had a significantly higher muscle weight after retraining by overload in the overloaded leg compared to the contralateral control leg (figure 4.10 B). The memory rats had a larger relative increase in muscle weight (31 %) compared to the control rats (21 %), consistent with the larger relative increase in CSA in memory rats (figure 4.8 D). Figure 4.10 C shows the connection between the CSA and the body weight of the rats. The rats with a higher body weight have a larger CSA. When the overloaded leg was normalized to its contralateral leg, the difference in body weight was accounted for, hence the relative difference displayed in figure 4.8 D provides a more valid comparison than the absolute difference in figure 4.8 C.

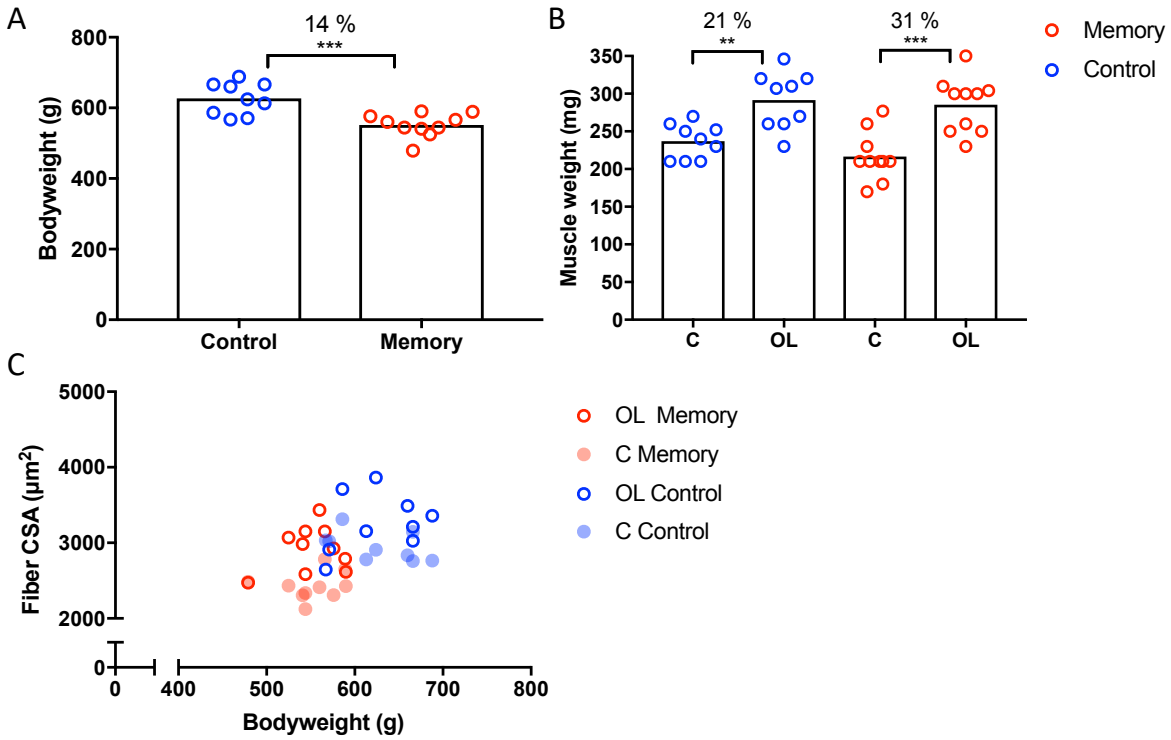


Figure 4.10 Control rats weigh more than previously trained rats upon retraining by overload
A) Body weight of the memory (red) and control (blue) rats after retraining by synergist ablation. Each dot represents a rat. ***: P = 0,0004. B) Muscle weight after retraining by overload. Weight of the right *soleus* undergoing overload (OL) and the left *soleus* not undergoing overload (C) in memory and control rats. ***: p = 0,0002, **: p = 0,0052. Each dot represents a muscle. *C*) The connection between the rat body weight and the cross-sectional area (CSA) of the overloaded and the contralateral control leg. Each dot represents a muscle average CSA and the respective rat body weight.

4.2.5 Changes in myonuclear domains are different in previously trained compared to previously untrained rats during retraining by overload

At the start of retraining by climbing, the memory rats had a significantly lower MND compared to controls, corresponding with figure 4.6 and 4.7. Retraining by climbing induced a decrease in the MND of both the memory (Pre RT: 964 ± 99, Post RT: 857 ± 60 μm^2) and the control group (Pre RT: 1156 ± 104, Post RT: 848 ± 50 μm^2). The difference was significant only in the control group (figure 4.11 A). Retraining by overload caused a significant increase in the MND of the memory group (C: 848 ± 41, OL: 975 ± 50 μm^2) and a maintained MND in the control group (C: 1079 ± 87, OL: 1055 ± 82 μm^2) (figure 4.11 B). The contralateral control leg of the memory and the control rats were significantly different after retraining by overload, corresponding with figure 4.8.

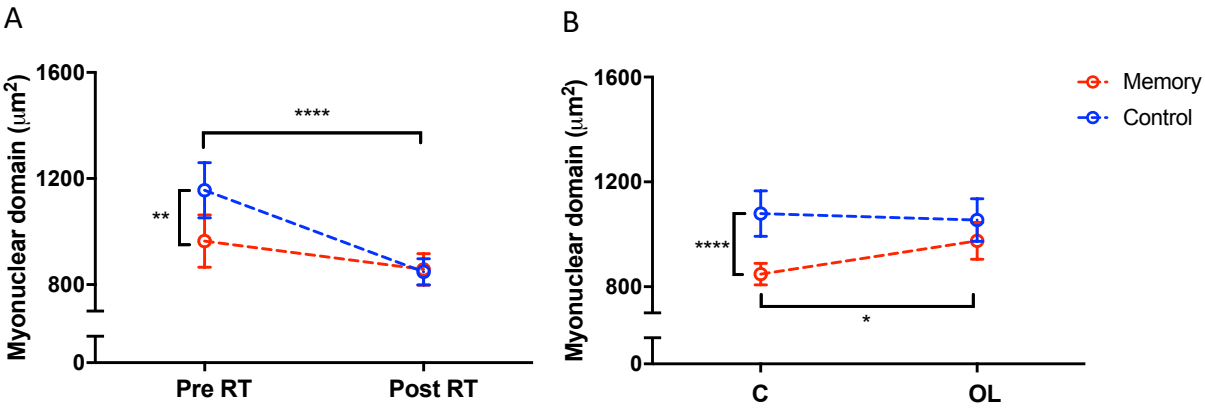


Figure 4.11 Myonuclear domains during retraining by climbing and overload
A) The myonuclear domain of memory (red) and control (blue) groups before and after retraining by climbing (RT). ****: P < 0,0001, **: P = 0,0026. B) Myonuclear domain in the overloaded groups and their contralateral control group after retraining by overload. *: P = 0,0209, ****: P = < 0,0001. Means ± 95 % CI.

5 Discussion

In this thesis we sought to investigate whether a cellular muscle memory mechanism, previously found in mice subjected to testosterone treatment (Egner et al., 2013), exists in humans and rats subjected to resistance exercise. We hypothesized that the elevated myonuclear number induced by training remains during long-term detraining and constitutes a cellular memory promoting subsequent hypertrophy. The human study could not be used to test the muscle memory hypothesis, as there was no increase in myonuclear number after the initial training period. The same memory hypothesis was tested in rats subjected to training by climbing, detraining, and subsequently retraining by climbing or synergist ablation. These results support the cellular memory hypothesis by showing that previously trained rats have an increased hypertrophic response to retraining, compared to previously untrained rats.

5.1 Human muscle memory project

5.1.1 No increase in myonuclear number during the initial training period

The initial training period did not lead to an increase in the number of myonuclei according to the single fiber analysis (figure 4.1 A-B, 4.2). Cross-sectional analysis done at NIH (unpublished data) showed the same lack of myonuclear addition. Their results strengthen the presented result, indicating that the lack of an effect is not due to technical weaknesses with the fiber analysis.

The findings in the present human study are supported by previous strength training studies done in humans. One study shows that 6 weeks of traditional strength training failed to induce a significant increase in myonuclear number in young sedentary females (Herman-Montemayor et al., 2015). A more recent study reports no significant changes in myonuclear number in young active males after 10 weeks of resistance exercise (Damas et al., 2018b). Further Kvorning *et al.* (2015) report that 8 weeks of resistance exercise in young active males caused a significant increase in myonuclear number in type 2, but not in type 1 fibers (Kvorning et al., 2015). Another study in young sedentary males reports a significant increase in myonuclear number in type 1 and type 2 fibers after 12 weeks of resistance exercise, but only in type 2 fibers after 8 weeks (Snijders et al., 2016). These previous studies indicate that the 10 weeks of resistance exercise used in the current study may not be sufficient to induce

significant increases in myonuclear number, at least not in type 1 and type 2 fibers together. To ascertain adequate satellite cell response and incorporation of myonuclei, 12 weeks should be considered a minimum for an initial training period in humans.

A physiological explanation for the lack of myonuclear addition during the first training period might be the proposed myonuclear domain threshold (Kadi et al., 2004). Previous human studies have indicated that hypertrophy up to 15-26 % might occur without incorporation of new myonuclei, indicating an ability in the existing myonuclei to support the growing cytoplasmic volume, at least up to this hypertrophic threshold (Bazgir et al., 2017; Kadi et al., 2004; Petrella et al., 2006). The increase in fiber volume in the present study was 12 % (figure 4.1 D), supporting the previous literature. These results imply that the subjects' muscle fibers contained a myonuclear number that still had not maximized their myonuclear domain capacity. As the subjects in the present study are classified as currently sedentary, they might have been more active when they were younger, thus containing a surplus of myonuclei that were able to support the small increase in fiber volume.

Purely methodological issues with the biopsy procedure should also be mentioned. A single biopsy has proven to be a modest estimator of the muscle fiber CSA for a whole human muscle because of the large variability in fiber CSA within a muscle (Lexell and Taylor, 1989). The biopsy sample may give false high or low values not representative for the actual values in the whole muscle. Obtaining more than one biopsy from the same muscle have shown to reduce sampling error more than analyzing a larger number of fibers from the same biopsy sample (Lexell et al., 1985). But due to ethical considerations and the large total number of biopsy samples, only one sample was taken from each muscle at each time point. Sampling from only one single part and depth of the muscle may include a selection of fibers with a certain fiber size, myonuclear number or fiber type proportion (Lexell et al., 1985), which might affect the results. Fibers expressing slow MyHC have been found to contain a higher amount of myonuclei than fast fibers, while fibers expressing fast MyHC have a larger fiber size (Allen et al., 1999; Liu et al., 2009), and an increased ability to undergo hypertrophy in response to resistance exercise (Kvorning et al., 2015; Snijders et al., 2016; Staron et al., 1991). Due to time limitations, we did not assess the fiber types of the single fibers through SDS PAGE (section 1.1.1) (Murach et al., 2016), but if the different biopsies did include a larger proportion of a certain fiber type, this might explain the lack of detected increase in myonuclear number and fiber volume, and the large inter-individual variation.

As our study required timeline analysis of the myonuclear number in live humans, a muscle biopsy sample was required. In a recent published study suggesting an epigenetic muscle memory, dual X-ray absorptiometry (DEXA) was used to measure body composition (Seaborne et al., 2018). After 7 weeks each of training, detraining and retraining, the authors found a significant increase in lower limb muscle mass after the retraining period compared to the training period, supporting the muscle memory hypothesis (section 1.5.1). Measuring body composition with DEXA is a much easier approach to measure muscle mass compared to measuring single fiber volume, but the myonuclear content is not possible to study through this approach.

5.1.2 Inter-individual variation might mask a true increase in fiber volume

The initial training period caused a 12 % increase in fiber volume (figure 4.1 D). This increase was not significant, possibly due to the large inter-individual variation (figure 4.1 C). When dividing the subjects based on gender, the males (N = 9) experienced a larger fiber volume increase (23 %) compared to all subjects pooled, indicating that the inter-individual variation might mask a potential biological effect in fiber volume. However, this increase was still not significant ($p = 0,09$). In order to detect effects from the inter-individual variation, a larger number of subjects should be included in the study.

In contrast to the non-significant increase in males, the fiber volume in females was unchanged after the initial training period (figure 4.3 B). Existing literature propose that males experience greater absolute muscle size gains compared to females after resistance exercise (Hubal et al., 2005; Ivey et al., 2000a; Ivey et al., 2000b; Kosek et al., 2006), as they originally have a larger absolute muscle and fiber volume at baseline (Janssen et al., 2000; Kosek et al., 2006). However, studies have found that the relative gains in muscle size are similar between the genders (Abe et al., 2000; Hubal et al., 2005), which is not consistent with the present study. The inconsistency in the present study might be explained by the N being too low to completely distinguish the gender-dependent response from a random inter-individual response.

Response to resistance exercise varies widely amongst individuals, and terms as non-, low-, and high-responders have been suggested (Bouchard et al., 2011; Hubal et al., 2005; Thalacker-Mercer et al., 2013; Vellers et al., 2018). In a previous study the subjects that

increased their muscle CSA less than 5 % during a 12-week resistance exercise period, were classified as non-responders (Hubal et al., 2005). The potential danger in this is sorting subjects into different groups based on their response, not knowing if these "non-responders" are simply "not-yet-responders" experiencing a delayed response. Hence the existence of non-responders has been debated, and several researchers claim that non-responders do not exist (Alvarez et al., 2017; Barbalho et al., 2017; Churchward-Venne et al., 2015). Other factors such as exercise adherence, activity outside the exercise regime, diet, rest and sleep, might in addition impact the variation in inter-individual response to the exercise regime in the present human study.

The variation in inter-individual response to the resistance exercise protocol in the present study is consistent with previous human studies. Gains in muscle CSA have been reported to vary from a 40 % increase to a 5 % decrease (Hubal et al., 2005). In the present human study changes in fiber volume during the initial training period varied from a 94 % increase to a 48 % decrease (figure 4.1). The larger range compared to the previous study might be due to measurements on single fiber volume and the accompanied variance discussed earlier related to e.g. biopsy variability instead of measuring the whole muscle size. The biopsy variability may also mask the actual effect in fiber volume. If the biopsies were composed of a larger proportion of type 1 than type 2 fibers compared to the real fiber type composition in the whole muscle, the actual increase in fiber volume might not be detected.

5.1.3 No effects of the retraining period

The retraining period caused no increase in myonuclear number or fiber volume (figure 4.1). As the retraining period was half the length of the initial training period, which did not increase the number of myonuclei, 5 weeks is most likely too short to induce myonuclear addition. To our knowledge there are no other studies investigating changes in myonuclear content in humans after training, detraining and retraining, thus we have no data to compare the present study with. Nonetheless, there are a few studies that have studied the changes in fiber size or muscle size during retraining. In the previously mentioned study by Seaborne *et al.* (2018) young sedentary males significantly increased their lean lower limb mass after 7 weeks of training. 7 weeks of detraining reverted this increase, and subsequent 7 weeks of retraining significantly increased the muscle mass more than previously trained state, supporting the muscle memory hypothesis. The present study failed to detect any changes in

fiber volume after 5 weeks of retraining. Discrepancies is probably due to the different muscle mass measurements, whereas effects on the single fiber level are harder to detect than on the whole lower limb level, because of the mentioned confounding factors.

Another study found significant increases after 6 weeks of retraining in young sedentary females in the CSA of type 2A, 2X, and 2AX hybrid fibers, but not in type 1 fibers (Staron et al., 1991). The control group in this study experienced similar gains in CSA, hence there are no confirmation of a muscle memory. However, this study reports to have excluded atrophic fibers, hence potentially overestimating the hypertrophic response, as well as the number of analyzed fibers varies amongst the different fiber types. Further, Staron *et al.* (1991) show that even a detraining period of 32 weeks did not completely revert the fiber size gain from the previous 20-week training period. The presented single fiber results indicate that the fiber volume is almost reverted to pre-trained state after 20 weeks of detraining (figure 4.1 C-D). However, the fiber CSA measurements provided from NIH showed no decrease in fiber CSA after the detraining period (unpublished results). This indicates that for future studies on muscle memory, detraining periods of up to a year could be considered to ascertain complete reversal of the fiber size (Correa et al., 2016). Additional muscle memory studies have been comprised of longer retraining periods than the present study (8-12 weeks), but with older subjects and no validation of muscle memory (section 1.5.1) (Correa et al., 2016; Taaffe et al., 2009; Taaffe and Marcus, 1997).

Other than the detraining and retraining period being too short, the potential effects of motor learning could be leading to further bias. When the resistance exercise protocol was designed, the unilateral approach was hypothesized to reduce the effect of motor learning, as both legs would learn the exercises during the initial training period. Thus, when subjected to retraining, the contralateral leg would also be familiar with the exercises (Lee and Carroll, 2007). Since it was not trained during the initial training period, the control leg was assumed to remain constant and was not biopsied, which was also partly due to ethical considerations. Hence, we were not able to investigate to what extent any potential contralateral effect affected the muscle cells of the contralateral control leg. To decrease the potential effects of motor learning, the adaptation phase prior to study initiation can for future reference be extended, at least since neural adaptations play an important role in early-phase adaptations in untrained subjects (Andersen et al., 2005; Hakkinen et al., 2000). Nonetheless, the

adaptation phase should not be extended too much, considering the risk of early-phase hypertrophy (Counts et al., 2017) occurring before the first biopsy is taken.

The strength tests performed at GIH indicated a muscle memory after retraining (unpublished data) while the presented cellular changes in the muscle did not (figure 4.1). These differences in strength and fiber volume results might be explained by motor learning. However, it might also be due to the mentioned confounding factors of the present human study; such as biopsy problems, large inter-individual variations and differences in response. The strength tests may be a more sensitive measurement where the confounding factors of the cellular measurements are absent. In that case those results would support the human muscle memory hypothesis.

5.2 Rat muscle memory project

5.2.1 A higher food intake and body weight in untrained rats

During the 5 first weeks of the rat climbing study, the trained rats had to climb to eat. This led to a higher activity level, a lower food intake and a lower body weight compared to the control group. Interestingly, the previously trained rats continued to eat slightly less than the previously untrained rats, even after they were back in normal cages during the detraining period (figure 4.4 A). After 10 weeks of detraining, this resulted in a significantly different body weight between the memory and the control group (figure 4.10 A). All though the observation that previously trained rats continued to eat less than previously untrained rats is an interesting finding in itself and might have public health implications, it is a confounding factor for the results of the present study. As the control rats weighed more, it induced a larger load on the *soleus* muscle during the study protocol, resulting in a larger muscle weight in the control rats compared to the memory rats prior to the retraining period (figure 4.5 B, 4.10 B). In retraining by climbing, this difference was avoided, as rats with similar body weights were selected for retraining, while rats with different body weights were used for less critical control groups not undergoing retraining (figure 4.4 A) (section 3.2.2). In order to reduce the number of animals in the experiment, there were no control rats not undergoing overload during retraining by overload. The controls used in this part of the study was the contralateral control legs. Hence, there were no possibility of selecting rats with similar body weight for retraining by overload. The body weight between the memory and the control rats is therefore significantly different in retraining by overload (figure 4.10 A), but not in retraining by climbing (figure 4.4 A). The control rats in retraining by overload that had a higher body weight also had a larger muscle weight (figure 4.10 B) and muscle fibers with larger CSA compared to memory rats (figure 4.10 C). Therefore, the absolute value of the CSA of the control rats is higher than the memory rats after retraining by overload (figure 4.8 C).

Furthermore, the initial training period caused a lower content of intra-abdominal fat in the trained group (figure 4.5 A). This decrease in fat content was greater than the decrease in body weight (figure 4.4 A). After retraining by climbing, the memory group had a lower content of intra-abdominal fat compared to the control group (figure 4.5 A), indicating that the previously trained rats have an ability for a more rapid fat loss during the retraining

period. This might be due to a motor learning effect, as the previously trained rats were familiar with the climbing cage in contrast to the previously untrained rats, thus able to resume a higher physical activity level during the first phase of the retraining period. This is supported by a higher food intake in previously trained rats compared to previously untrained rats during the first week of retraining by climbing, indicating that the memory group climbed more than the control group (figure 4.4 B). There was no difference in muscle weight between the two groups after the training or retraining periods (figure 4.5 B), which might be caused by an increased amount of intra-myocellular fat in the *soleus* muscle accompanied by the increased intra-abdominal fat in the control rats (figure 4.5 A) (Saukkonen et al., 2010).

5.2.2 Myonuclei are not lost during atrophy

Training by climbing induced a significant increase in myonuclear number and a non-significant increase in fiber CSA in trained compared to untrained rats (figure 4.6). The elevated number of myonuclei was still maintained after detraining, even though the CSA was back to baseline (figure 4.7). This indicates that myonuclei are not lost during atrophy, which supports existing literature (Bruusgaard et al., 2010; Egner et al., 2013; Schwartz et al., 2016; Wada et al., 2002). The elevated myonuclear number induced by climbing remained even after 10 weeks of detraining which corresponds to 5 human years (Sengupta, 2013), supporting the proposed long-lasting effect of the cellular muscle memory (Egner et al., 2013; Gundersen, 2016). For a given CSA, the previously trained rats had a higher myonuclear number than the previously untrained rats (figure 4.7), providing a basis for studying our cellular memory hypothesis, in contrast to the human study (figure 4.2).

5.2.3 Climbing fails to induce a significant increase in fiber size

Incorporation of myonuclei has previously been shown to precede hypertrophy, suggesting that the higher total protein synthetic capacity is needed to increase the fiber size (Bruusgaard et al., 2010), which the present study supports (figure 4.6). Even though there were significant increases in the number of myonuclei, there were no significant changes in CSA neither after training or retraining by climbing (figure 4.6 C). Jung *et al* (2015) report a significant increase in fiber diameter of the quadriceps after 8 weeks of ladder-climbing exercise, and a significant increase in muscle CSA of the *flexor halluces longus* (*FHL*) was

induced after 8 weeks in another study (Lee et al., 2004). The climbing protocol in the present study failed to reach similar CSA gains, which might be connected to the fact that no additional load was added to the climbing rats. In the mentioned studies weight was attached to the tail of the rat and loads up to 100 % of the rats body weight was added each set until the rat could no longer climb the entire length of the ladder (Jung et al., 2015; Lee et al., 2004; Lee et al., 2016; Yarasheski et al., 1990). As the rats increase their body weight over the time course of the climbing study (figure 4.4 A) this might be considered as an increased load, however a low one compared to previous studies. Low load has proven to cause equivalent hypertrophy compared to high loads when applied to failure (Mitchell et al., 2012; Ozaki et al., 2016). The rats in the present study was not forced to climb by electric shocks to the tail as rats in previous studies have been (Jung et al., 2015; Lee et al., 2004). As starvation will only motivate an animal to exert 50-60 % of its maximal voluntary capacity, the rats most likely stopped climbing before they reached failure (Cholewa et al., 2014). Hence, the climbing protocol in the present study might have failed to provide sufficient stimuli required for significant hypertrophy either through the lack of a progressively increased load or through not reaching failure, or both. In addition, the rat *soleus* muscle is predominantly composed of type 1 fibers (Armstrong and Phelps, 1984; Liu et al., 2009), shown to increase less in fiber size compared to type 2 fibers in response to resistance exercise (Yarasheski et al., 1990). Thus, a muscle with a higher proportion of type 2 fibers, such as *tibialis anterior* or *FHL* (Armstrong and Phelps, 1984) could be considered for future perspective.

Retraining for 2 weeks by climbing did not induce a significant increase in CSA in any of the groups (figure 4.6 C). As 5 weeks of climbing was not sufficient to cause significant fiber size changes, retraining for only 2 weeks would not be expected to differ. Previous rat climbing studies have used 7 or 8 weeks as training (Deschenes et al., 1994; Jung et al., 2015; Lee et al., 2004; Lee et al., 2016; Yarasheski et al., 1990) and retraining periods (Lee et al., 2016). For future cellular muscle memory investigations in rats by climbing, retraining periods should be extended if the same climbing protocol as presented is used. Potentially beneficial for the hypertrophic response is a way of adding load throughout the climbing study to increase the applied stimuli (Cholewa et al., 2014; Marcotte et al., 2015; Schoenfeld, 2010). Lee *et al.* (2016) studied changes in muscle CSA of *FHL* after training by ladder-climbing exercise for 8 weeks, detraining for 20 weeks and subsequent retraining for 8 weeks, and report a significant increase greater after retraining in previously trained rats compared to controls, supporting a muscle memory mechanism (Lee et al., 2016). In

summary discrepancies in the detected hypertrophic response compared to the present study might be due to differences in; climbing protocols, lengths of retraining periods, muscle examined, and CSA measurements on the whole muscle rather than on fiber cross sections.

Aging impairs satellite cell function (Sousa-Victor et al., 2015; Verdijk et al., 2014). We wanted to maximize the muscle fibers ability to add myonuclei and undergo hypertrophy, thus periadolescent rats (4-weeks-old) were chosen for the present climbing study. However, this decision introduced another confounding factor. Post-natal growth in rats include hypertrophy and an increase of myonuclear number (Enesco and Puddy, 1964), hence the measured parameters in the present study increased independently of the intervention as the rats grew. Especially during the first weeks of post-natal life (Sengupta, 2013), which is apparent in figure 4.6 A and C where the absolute values of the myonuclear number and CSA have increased in both groups after 10 weeks of detraining. Old and young rats are proposed to respond differently to ladder-climbing exercise. Jung *et al.* (2015) report that 50-weeks-old rats did not significantly increase their muscle fiber diameter after 8 weeks of ladder-climbing exercise, while 10-weeks-old rats did (Jung et al., 2015). The rats in the present study are 21-weeks-old at the oldest, hence a similar decrease in response will most likely not occur and does thus not explain the lack of significant hypertrophy during retraining by climbing.

5.2.4 Previously trained rats grow twice as much as previously untrained rats during retraining by overload

Retraining by climbing caused a non-significant increase of 7 % in the CSA of previously trained compared to previously untrained rats (figure 4.6 C-D). Retraining by overload induced a significant increase in CSA in both groups (figure 4.8 C-D), consistent with previous studies (Goldberg, 1967; Nakada et al., 2016; Snow, 1990). Overload induced an increase in CSA of 20 % in the previously trained rats, which was almost twofold the increase in previously untrained rats which was 11 % (figure 4.8 D, 4.9). This is consistent with the muscle weight of the contralateral and overloaded leg of the memory and control rats, where overload induced an increase in muscle weight of 31 % in the previously trained rats, while the previously untrained rats only increased by 21 % (figure 4.10 B). So, even though the previously untrained rats started at a higher absolute value of CSA and muscle weight, the relative increase of CSA and muscle weight after retraining by overload was

larger in previously trained rats compared to previously untrained rats (figure 4.8 D, 4.10 B). This indicates that the elevated myonuclear number acquired through training by climbing (figure 4.7) promoted an increased ability for fiber growth, supporting the cellular muscle memory hypothesis (Egner et al., 2013; Gundersen, 2016).

5.2.5 Less myonuclear addition in previously trained rats

Retraining by climbing and overload both indicates less myonuclear addition in previously trained rats compared to previously untrained rats (figure 4.6 A-B, 4.8 A-B). After retraining by climbing, the difference in myonuclear number between the two groups had decreased and were no longer significant (figure 4.6 A-B). Retraining by overload showed a significant difference in myonuclear number in the previously untrained rats, but not in the previously trained rats (figure 4.8 A-B). Altogether, these results support our cellular muscle memory hypothesis, as the existing myonuclei in previously trained muscles have an increased ability to support a growing cytoplasmic volume thus not the same need to add new myonuclei. The difference in MND after retraining by overload also visualizes that there was less myonuclear addition in previously trained rats (figure 4.11 B). Retraining by overload caused an increase in the MND of the overloaded leg compared to the contralateral leg in previously trained rats, while in previously untrained rats the MND decreased. Increased MND in previously trained rats was a result of a significant fiber size gain (figure 4.8 C) and a non-significant increase in myonuclear number (figure 4.8 A). Therefore, the preexisting myonuclei needed to support a larger MND. The significant increase in CSA (figure 4.8 C) combined with the significant increase in myonuclear number (figure 4.8 A) resulted in a decreased MND in previously untrained rats, providing additional myonuclei to support the growing cytoplasmic volume during these muscles' first encounter with training. The same changes are not apparent in retraining by climbing (figure 4.10 A), as the climbing protocol failed to induce a significant increase in CSA in both groups (figure 4.6 C).

Taken together, these findings support the previous studies from our lab suggesting that it does not appear to be possible to 'pump up' the muscle fiber with myonuclei through repeated hypertrophy and atrophy cycles (Bruusgaard et al., 2012; Egner et al., 2013; Gundersen, 2016). As there is less myonuclear addition in previously trained muscles, the increased number of myonuclei appears to represent the largest size the fiber has had in its history – a memory of previous trained state.

6 Conclusion

In this thesis I provide evidence for a cellular memory mechanism in the skeletal muscles of rats subjected to resistance exercise. Our results show that myonuclei acquired through training by climbing remains during long term detraining, indicating that muscle memory may have long-lasting effects also under more physiological relevant conditions. Previously trained muscles that contained an elevated number of myonuclei showed increased hypertrophy in response to retraining by overload compared to previously untrained muscles. The presented human study turned out to be unsuitable for testing the cellular muscle memory mechanism due to a lack of myonuclear addition during the first training period. Even though the study failed to confirm a cellular muscle memory, it does not provide evidence against such a hypothesis either. Other studies are needed to delineate the existence of a cellular muscle memory mechanism in humans.

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7 Appendix

7.1 Appendix 1: Solutions

Section	Solution	Ingredients	Volume
3.1.3	40 % NaOH		100 mL
		Solid NaOH	4 g
		dH_2O	100 mL
3.1.3	10 X PBS		1 L
		NaCL	80.0 g
		KCl	2.0 g
		Na_2HPO_4 x $2H_2O$	14.4 g
		KH_4PO_4	2.0 g
		dH_2O	1 L
3.2.3	1 X PBS		1 L
		PBS (10X)	100 mL
		dH_2O	900 mL
3.2.3	5 % BSA in PBS		10 mL
		BSA powder	50.0 mg
		PBS	10 mL
3.2.3	2 % BSA in PBS		10 mL
		BSA powder	20.0 mg
		PBS	10 mL

7.2 Appendix 2: Reagents

Section	Name	Supplier	Product number
3.1.3, 3.2.3	DAPI Fluoromount-G ®	Southern Biotech	0100-20
3.2.2	Tissue-Tek OCT matrix	Sakura	REF4583
3.2.3	Igepal ® CA-630	Sigma-Aldrich	I8896
3.2.3	Anti-PCM1, primary antibody produced in rabbit	Sigma-Aldrich	HPA023370 Batch: B114638
3.2.3	Secondary Antibody, Goat Anti-Rabbit, Alexa 488	Abcam	AB150077
3.2.3	Anti-Dystrophin, primary antibody produced in mouse MANDYS8	Sigma-Aldrich	D8168
3.2.3	Secondary Antibody, Goat Anti-Mouse, Alexa 594	Thermo Fisher Scientific	A-11005