Na^+, K^+-ATPase in single skeletal muscle fibres and the effects of ageing, training and inactivity

Submitted by

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ABSTRACT

The Na^+,K^+-ATPase (NKA) is a key protein involved in the maintenance of skeletal muscle excitability and comprises 2 subunits (α and β), each of which express multiple isoforms at a protein level in skeletal muscle (α_{1-3} and β_{1-3}). The fibre-specific expression, adaptability and roles of each isoform in human skeletal muscle are explored in this thesis. Research utilising muscle biopsies typically uses samples obtained from the vastus lateralis muscle, which in healthy young people comprises similar proportions of type I and II fibres. Analyses using whole muscle pieces don't allow the detection of fibre-type specific differences and changes occurring at a cellular level. Hence analysis of skeletal muscle samples at the single fibre level offers important advantages in understanding NKA regulation. This thesis therefore investigated the isoform abundance of the NKA in human skeletal muscle single fibres and their adaptability following intense repeated-sprint exercise (RSE) training in healthy young adults (Study 1); with ageing (Study 2) and after high-intensity interval training (HIT) in the elderly (Study 3) and after voluntary inactivity and resistance training in healthy young adults (Study 4).

Study 1. The NKA plays a key role in muscle excitability, but little is known in human skeletal muscle about possible fibre-type specific differences in NKA isoform expression or adaptability. Hence a vastus lateralis muscle biopsy was taken in 17 healthy young adults and the NKA isoform protein relative abundance contrasted between type I and II fibres. The muscle fibre-type specific NKA adaptability in eight of these adults was then investigated following four weeks of repeated-sprint exercise (RSE) training, comprising three sets of 5x4-s sprints, three days/week. Single fibres were separated and myosin heavy chain (MHC I, MHC II) and NKA (α_{1-3} and β_{1-3}) isoform abundance were determined via western blotting. All six NKA isoforms were found to be expressed in both type I and II muscle fibres;

however, only the β_2 NKA isoform exhibited fibre-type specific differences in abundance. RSE training increased the β_1 in type I fibres only (Pre-Train 0.58±0.28, Post-Train 0.76±0.38 a.u., 30%, $p<0.05$), but no training effects were found for other NKA isoforms in either type I or II fibres. Thus human skeletal muscle does not express NKA isoforms in a fibre-type specific manner and co-expression of all six NKA isoforms points to their different functional roles in skeletal muscle cells. The detection of elevated NKA β_1 abundance in type I fibres following training, may have implications for increasing NKA activity during intensive exercise, but needs further investigation. Finally, the fibre-type specific upregulation of β_1 demonstrates the sensitivity of the single fibre western blotting technique for detecting fibre-type specific training effects.

Study 2. Ageing is associated with a reduction in muscle function and exercise performance, which may be attributed in part to alterations in isoform composition of the NKA and may include fibre type specific alterations. Therefore, 17 healthy older adults (69.4 ± 3.5 years, 170.8 ± 10.4 cm 75.2 ± 13.0 kg and 8.2 ± 4.4 hours of physical activity per week) and 14 younger adults (25.5 ± 2.8 years, 173.1 ± 12.3 cm, 72.9 ± 15.6 kg, 7.0 ± 3.9 hours of physical activity per week) underwent a resting muscle biopsy for measurement of NKA isoforms in single fibres and in a whole muscle homogenate (western blotting), as well as NKA content ([^{3}H]ouabain binding site content). Compared to young, older adults had 17 % lower α_3 NKA isoform abundance in type II fibres (1.17 ± 0.72 vs. 0.97 ± 0.79 a.u, $p < 0.05$), 41% lower β_2 isoform abundance in type I fibres (1.15±0.47 vs. 0.67±0.50, $p < 0.05$) and β_3 increased in type I fibres ($p<0.05$) There was a tendency for β_2 to be decreased in type II fibres ($p=0.09$) compared to young. No differences were detected in whole muscle homogenate for any of the isoforms, except for β_3, which conversely to single fibre analysis was decreased. The NKA content did not differ between young and old (341±59.8 vs. 362±57.5 pmol.g^{-1} wet weight). The NKA isoform expression differed with age in both type I and II fibres, with lower α_3 and

β_2 in the elderly. This study showed that older adults matched for physical activity to healthy young adults had no differences in the relative abundance of key NKA α_1, α_2 and β_1 isoforms or in NKA content. It is possible that physical activity may be more important to NKA content and not age per se. However, older adults did have decreases in the relative abundance of α_3 and β_2 isoforms compared to young and the decline in these isoforms may play a role in decreasing muscle NKA activity.

Study 3. Healthy young adults typically show increased skeletal muscle NKA content in response to exercise training, which usually coincides with improved exercise performance. Whether this is true in older adults and the NKA isoform abundance changes at the single fibre level are also unknown. Fifteen older adults (study 3) were randomised into either a control (CON, n=7) or training group (HIT, n=8) which underwent 12 weeks of high-intensity interval training (4x4 min at 90-95% peak heart rate) for 3 days /wk. Participants underwent a resting muscle biopsy prior to (Pre) and 48-72 hr following the final training session (Post) for measurement of NKA isoforms in single fibres and in a whole muscle homogenate (western blotting) and NKA content ($[^3H]$ouabain binding site content). An incremental cycling exercise test was also completed before and after training. In HIT, both VO_2 peak (18%) and peak power (25%) were improved following training ($p<0.05$), with no changes in CON. In single fibres, after HIT, the NKA α_2 isoform relative abundance was increased by 30% in type II fibres (Pre 0.69 ± 0.25 vs. Post 0.90 ± 0.40 a.u, $p<0.05$) β_2 abundance was increased by 52% in type I fibres (Pre 0.75±1.14 vs. Post 1.14±0.65 a.u , $p<0.05$) and β_3 isoform was decreased by 48% in type I fibres (Pre 1.44±1.10 vs. Post 0.74±0.44 a.u, $p<0.05$). Whole muscle analyses showed no change in α_2 or β_2 isoform abundance after HIT. The mitochondrial protein COX IV measured in whole muscle homogenate was increased by 19% after HIT ($p<0.05$). The NKA content tended to be higher after HIT (Pre 369.8 ± 52.7 vs. Post 403.0 ± 66.0, pmol.g wet weight^{-1}, $p<0.07$), with no

change in CON. This short volume, high-intensity training protocol was effective in improving fitness in the elderly, increased muscle mitochondrial proteins and tended to increase skeletal muscle NKA content. Furthermore, the use of whole muscle homogenate was unable to detect changes in NKA isoforms after HIT that were evident on a cellular level, indicating the single fibre technique should be utilized to detect potential training effects.

Study 4. Physical inactivity contributes to the development of numerous diseases, poor muscle function and health. Induced inactivity via unilateral lower limb suspension (ULLS) has detrimental effects on skeletal muscle fibre size, strength and function. The effects of ULLS on skeletal muscle NKA, which is important to membrane excitability, is unknown. Therefore this study investigated the effects of 23 days of ULLS on NKA isoform abundance in single muscle fibres from six healthy sedentary adults (4 males, 2 females; age: 22.4± 2.1 yr, mass: 71.3 ± 14.3 kg, height: 175.0 ± 11.7 cm, BMI: 23.2 ± 3.6 $kg.m^{-2}$, $\dot{V}O_2$ peak 45.5 ± 5.8 $ml.kg^{-1}.min^{-1}$, mean ± SD). Participants walked on crutches for 23 days, wearing a shoe on their right foot with an enlarged sole (10 cm), thus enabling the left leg to hang freely and be unloaded. Participants subsequently underwent 4 weeks of resistance training (RT). Muscle biopsies were collected from the left vastus lateralis muscle before (CON) and after ULLS (ULLS) and after RT and single fibres were collected and analysed. Compared to pre, 23 days of ULLS decreased α_3 abundance in type I fibres (CON 1.70 ± 0.68, ULLS 1.26 ± 0.88 a.u, $p<0.05$) and this was not restored by RT (ULLS 1.26±0.88, RT 1.09±0.26 a.u). There was a tendency for α_1 ($p<0.09$) and β_2 ($p<0.06$) to be increased in type I fibres following ULLS. Surprisingly, the NKA isoforms α_1, α_2 and β_1 were not different following ULLS. Thus, short-term unloading via ULLS induced fibre-type specific adaptability of the NKA isoforms, including a decreased α_3 and tendency to increase α_1 and β_2 abundance in type I fibres. The lack of a negative effect of unloading on most NKA isoforms was surprising and a longer duration of ULLS may be required to see more dramatic changes. The

functional effect of the increase α_1 and β_2 isoforms may be to increase NKA activity, but this remains to be determined. Furthermore, 4 weeks of RT was unable to restore the α_3 isoform and it is likely the decrease of α_3 was too severe to be returned to baseline from only four weeks of training.

In conclusion, this thesis has shown the co-expression of all six NKA isoforms within a human single skeletal muscle fibre and this was shown in both type I and II fibres. Additionally, the fibre-type specific adaptability of these isoforms in human skeletal muscle was demonstrated following both RSE training and unloading in healthy young adults, as well as fibre-type adaptability of the NKA in ageing and following HIT. Further, this thesis shows that after HIT, an increase of α_2 isoform relative abundance in type II fibres coincided with a tendency of greater [^{3}H]ouabain binding in older adults.

The interventions used in this thesis resulted in different outcomes for the NKA isoforms within both type I and II fibres. The key NKA isoforms in skeletal muscle (α_1, α_2 and β_1) were hardly affected or not affected at all by RSE training, ULLS, ageing or HIT in the elderly. The functional effects of these differences in NKA isoform expression and adaptability on NKA activity and skeletal muscle excitability are a direction for future research.

DECLARATION

I, Victoria Louise Wyckelsma, declare that the PhD thesis entitled *Na^+, K^+-ATPase in single skeletal muscle fibres and the effects of ageing, training and inactivity* is no more than 100,000 words in length, exclusive of tables, figures, appendices, references and footnotes. This thesis contains no material that has been submitted previously, in whole part, for the award of any other academic degree or diploma. Except where otherwise indicated this PhD is my own work.

Signature Date

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ABBREVIATIONS

[ ]	Concentration
Ca^{2+}	Calcium ion
K^+	Potassium ion
$[K^+]$	Potassium ion concentration
$[K^+]_a$	Arterial potassium ion concentration
$[K^+]_I$	Interstitial potassium ion concentration
Na^+	Sodium ion
$[Na^+]$	Sodium ion concentration
$[Na^+]_I$	Interstitial sodium ion concentration
Mg^{2+}	Magnesium ion
[^{3}H]ouabain binding	Tritiated-ouabain binding
α	Alpha
β	Beta
µg	Microgram
µl	Microlitre
ADP	Adenosine diphosphate
AP	Action potential
ATP	Adenosine 5´ triphosphate
BMI	Body mass index
CaMKII	Ca^{2+} calmodulin-dependent protein kinase
cAMP	Adenosine 3´,5´-cyclic monophosphate
CGRP	Calcitonin gene related peptides
CO_2	Carbon dioxide
COX IV	Cytochrome c oxidase/ complex IV
DHPR	dihydropyridine receptor
DVT	Deep vein thrombosis
EDL	Extensor digitorum longus muscle
E_m	Membrane potential
FXYD1/PLM	Phospholemman
FXYD5	FXYD-domain containing ion transport regulator 5
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

HIT	High-intensity training
IGF-1	Insulin growth factor-1
kDa	Kilodalton
kg/m^2	Kilogram per meter squared
MCT1	Monocarboxylate transporter 1
MCT4	Monocarboxylate transporter 4
MHC	Myosin heavy chain
mRNA	Messenger ribonucleic acid
Na^+, K^+-ATPase/NKA	Sodium, potassium adenosine 5´triphosphatase
Na-EGTA	Sodium-ethylene glycol tetraacetic acid
PGC1-α	Peroxisome proliferator-activated receptor gamma coactivated
PKA	Protein kinase A
PKC	Protein kinase C
$pmol.g^{-1}$	picomoles per gram
RG	Red gastrocnemius muscle
RPE	Rating of perceived exertion
RPM	Revolutions per minute
RSE	Repeated-sprint exercise
RyR	Ryanodine receptor
SCI	Spinal cord injury
SDS	Solubilising buffer
SOL	Soleus muscle
SR	Sarcoplasmic reticulum
t-tubules	Transverse tubules
ULLS	Unilateral lower limb suspension
$VO_{2\ max}$	Maximum oxygen uptake
$VO_{2\ peak}$	Peak oxygen uptake
$W.min^{-1}$	Watts per minute
WG	White gastrocnemius muscle
wk	Week

PUBLICATIONS AND PRESENTATIONS

This thesis is supported by the following conference presentations and publications

Publications

1. **Wyckelsma VL**, McKenna MJ, Serpiello FR, Lamboley CR, Aughey RJ, Stepto NK, Bishop DJ, Murphy RM. Single fibre co-expression and fibre-specific adaptability to short-term intense exercise training of Na^+, K^+-ATPase α and β isoforms in human skeletal muscle. *Journal of Applied Physiology*, Submitted April 2014.

Presentations

1. **Wyckelsma VL**, Levinger I, Petersen AC, Perry BD, Atanasovska T, Farr T, McKenna, MJ. K^+ dynamics in older adults during incremental exercise and recovery. Oral presentation, *MyoNaK (skeletal muscle and cardiac sodium- potassium pump meeting)* Beitostolen, Norway, 2012

2. **Wyckelsma VL**, Murphy RM, Serpiello FR, Lamboley CR, McKenna MJ. Repeated-sprint exercise training up-regulates the β_1 isoform of the Na^+-K^+-ATPase in both type I and IIa fibres in human skeletal muscle fibres. Oral Presentation. *European College of Sport Sciences (ECSS) Conference.* Barcelona, Spain, June 2013

3. **Wyckelsma VL**, Murphy RM, Serpiello FR, Lamboley CR, McKenna MJ. Fibre-type specific differences and adaptability of the Na^+, K^+-ATPase α_{1-3} and β_{1-3} isoforms to intermittent training in human single skeletal muscle fibres. Poster Presentation. *International Union Physiological Society (IuPS) Meeting.* Birmingham, United Kingdom, July 2013.

4. **Wyckelsma VL**, Murphy RM, Levinger I, Petersen AC, McKenna MJ. The effects of high-intensity interval training on aerobic power and skeletal muscle Na^+, K^+-ATPase in single fibres of healthy older adults. Oral Presentation. *Australian Society of Medical Research (ASMR) National Conference*. Ballarat, Australia, Nov 2013.

5. **Wyckelsma VL**, Murphy RM, Levinger I, Petersen AC, McKenna MJ. High-intensity interval training in older adults does not upregulate the Na^+, K^+-ATPase isoforms measured in whole muscle homogenate, but shows fibre type specific upregulation when analysed on the single fibre level. Oral Presentation. *Australian Physiological Society (AuPS) National Conference*. Geelong, Australia, Dec 2013

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CHAPTER 1. INTRODUCTION

Muscle contraction requires the repeated propagation of action potentials (AP) along and into the muscle cells, which is dependent on the cell membrane potential and excitability, which are regulated by intracellular and extracellular concentration gradients and conductance of K^+, Na^+ and Cl^- ions (Hodgkin & Horowicz, 1959; Pedersen *et al.*, 2005). During muscle contractions, increased AP frequency result in a greater exchange of Na^+ and K^+ ions across the cell membrane and may impair excitability (Sjøgaard *et al.*, 1985; Sejersted & Sjøgaard, 2000). The skeletal muscle Na^+, K^+-ATPase (NKA) enzyme counteracts the excitation-induced Na^+ and K^+ fluxes and thus is important in maintaining excitability and contraction. Consequently, any modulation of muscle NKA, either acutely or chronically, has the potential to affect muscle function. In muscle the NKA content is measured using the [^{3}H]ouabain binding site content assay, with NKA isoform expression measured by western blotting, typically in whole homogenates or in muscle extracts. In skeletal muscle, six isoforms (α_{1-3}, β_{1-3}) of the NKA are expressed. The basic function of each isoform is listed below in Table 1.1.

Table 1.1 Basic functions of the NKA isoforms in skeletal muscle

NKA isoform	Function
α_1	Key housekeeping isoform, large contribution to Na^+/K^+ exchange mainly during basal conditions
α_2	Key α isoform in skeletal muscle, comprises approximately ~80% of the α subunits. Major function is to Na^+/K^+ exchange during muscle contractions

α_3	Has a minor abundance in skeletal muscle, with its role not specifically known. Most abundant in neurons where it functions as a key Na^+/K^+ exchanger.
β_{1-3}	The roles of individual β subunits in skeletal muscle are yet to be investigated. The β subunit plays a regulatory role.

Skeletal muscle biopsy samples in humans are typically obtained from the vastus lateralis muscle, which comprises a similar proportion of type I and II fibres. Western blotting approaches use typically whole or fractioned muscle homogenate, which therefore contain a mixture of both type I and II fibres. The use of whole muscle has been effective in detecting general adaptability of the [^{3}H]ouabain binding site content and NKA isoforms following training, inactivity induced by injury and ageing (Leivseth & Reikeras, 1994; Bangsbo *et al.*, 2009; Boon *et al.*, 2012; Perry *et al.*, 2013). However a limitation of these methods is a lack of sensitivity towards the detection of any fibre type specificity in either the expression or adaptability of NKA. A recent study reported fibre-type specific expression of the NKA α_2 isoform and of phosphorylation of FXYD1 following acute intense exercise (Thomassen *et al.*, 2013). It is plausible that other NKA isoforms may also be expressed or adapt in a fibre-type specific manner following various interventions. It therefore would be advisable to undertake bio-molecular measurements of NKA isoforms in single skeletal muscle fibres to have a more complete understanding of the underlying molecular properties and adaptations of the NKA following training, inactivity and in ageing.

In animals, studies utilising isoform-specific antibodies have found the α_1 isoform to be equally abundant in oxidative and glycolytic muscles (Hundal *et al.*, 1993; Ng *et al.*, 2003; Fowles *et al.*, 2004), whereas numerous studies have shown similar abundance in both muscle types for NKA α_2 isoform (Hundal *et al.*, 1993; Fowles *et al.*, 2004; Kristensen &

Juel, 2010). In contrast the [^{3}H]ouabain site content, of which only measure the $\alpha_2$isoform in rat, is greater in fast compared to slow muscle (Clausen *et al.*, 1982). To date, the fibre-type specific expression of α_3 has not been investigated. Reports on NKA β isoform expression, using specific antibodies, consistently show fibre-specific differences, with a greater abundance of β_1 in oxidative muscle and β_2 in glycolytic muscle (Thompson & McDonough, 1996a; Fowles *et al.*, 2004; Zhang *et al.*, 2006); a similar abundance of β_3 has been reported in oxidative and glycolytic fibres (Ng *et al.*, 2003).

In human skeletal muscle, the fibre-type specificity of three of the six NKA isoforms has recently been investigated, with the α_1 and β_1 being similarly expressed in type I and II fibres, whilst the α_2 was found to be more abundant in type II fibres (Thomassen *et al.*, 2013). No other studies have investigated possible fibre-type specific expression of the other three isoforms (α_3, β_2, β_3). The expression of all NKA isoforms in skeletal muscle single fibres was investigated in Chapter 3.

Several of the NKA isoforms are adaptable to exercise training, with various high-intensity training protocols inducing upregulation of the key NKA α_1, α_2 and β_1 isoforms (Mohr *et al.*, 2007; Green *et al.*, 2008; Iaia *et al.*, 2008; Bangsbo *et al.*, 2009; Thomassen *et al.*, 2010; Benziane *et al.*, 2011). Typically, sprint training programs utilise exercise bouts of 30 s or longer, but the effects of a repeated-sprint exercise (RSE) training, consisting of repeated 4s sprints, replicating efforts produced in team sports are less understood. RSE is a critical fitness component for team sport athletes (Spencer *et al.*, 2005) and the effects of RSE training on NKA abundance are unknown. Given the intense intermittent nature of this training, presumably a large proportion of both type I and II fibres would be recruited; whether upregulation of NKA isoforms occurs in a fibre-type specific manner after RSE training was also investigated in Chapter 3.

Ageing is associated with a reduction of muscle function and exercise performance. This reduction might be partially attributed to a decline in NKA content and alterations in isoform composition and which may also include fibre-type specific alterations. A recent study has reported no difference in [^{3}H]ouabain binding site content between young and older adults but decreases in the abundance of NKA α_2 (24%) and β_3 (23%) isoforms in older adults compared to young (McKenna *et al.*,2012). Other NKA adaptations typically found in aged rodent muscle, such as increased α_1, β_1 and β_2 isoforms were not detected in whole muscle homogenate. It is possible that altered expression of these isoforms may occur in a fibre-type specific manner, and thus may not have been detected. The isoform abundance of NKA in single fibres and in a whole muscle homogenate along with muscle [^{3}H]ouabain binding were therefore investigated in both young and old healthy adults Chapter 4.

In healthy young adults, intense exercise training increases [^{3}H]ouabain binding site content and the relative abundance of several of the NKA isoforms (McKenna *et al.*, 1993; Nielsen *et al.*, 2004; Green *et al.*, 2008). Older adults that had been training between 12-17 years had a greater [^{3}H]ouabain binding site content than untrained older adults (Klitgaard & Clausen, 1989), but possible differences in isoform abundance were not measured. Aged rats who underwent a continuous running program with speed and time overload for 13-14 weeks exhibited fibre type-specific upregulation of the NKA α_1, α_2 and β_1 isoforms and decreased β_3 isoform following training, in both red and white gastrocnemius muscle (Ng *et al.*, 2003). However, the effects of a longitudinal training program on NKA isoform abundance in human are unknown. Therefore Chapter 5 investigated whether 12 weeks of high-intensity interval training (HIT) in aged individuals induced adaptations in NKA isoforms in a fibre-type specific manner, in a whole muscle homogenate, further [^{3}H]ouabain binding site content in whole muscle could be detected. .

Physical training increases the [^{3}H]ouabain binding site content in rat human and rat skeletal muscle, with high levels of physical activity typically associated with greater muscle NKA content (Murphy *et al.*, 2007). In contrast immobilisation decreased [^{3}H]ouabain binding in ovine skeletal muscle (Jebens *et al.*, 1995). Complete spinal cord injury reduced NKA α_1, α_2 and β_1 isoforms compared to able bodied controls when measured in a mixed fibre homogenate extract (Boon *et al.*, 2012). Unilateral lower limb suspension (ULLS) is an effective model for reducing muscle mass, strength and function in healthy people without a pre-existing injury (Berg *et al.*, 1991; Tesch *et al.*, 2004). This model enables research into inactivity without the complications of injury per se, as well as associated medications, hormonal imbalances and other issues that all must be considered when using injury as a model for inactivity. Therefore the final study of this thesis investigated the effects of short-term inactivity on the abundance of NKA isoforms were investigated in single fibres following 23 days of ULLS in Chapter 6.

CHAPTER 2- Literature Review

2.1 Overview of skeletal muscle structure

Skeletal muscle is a highly complex and precisely regulated tissue that is vital for posture and responsible for force generation and movement. Skeletal muscle can rapidly derive energy from fuel sources during repeated contractions, at a rate up to 300 fold greater than at rest and this can occur within milliseconds (Westerblad *et al.*, 2010).

The composition of skeletal muscle can be considered from a hierarchical perspective. To enable contraction muscle is fundamentally comprised of many thousands of individual muscle fibres/cells. The size of each individual muscle cell varies, but might be as small as one hundred microns in diameter up to 2-3 millimetres in length (Wells *et al.*, 2009). A varying number of fibres are bundled together and enclosed by connective tissue known as the perimysium; this bundle of fibres is known as a fascicle (Wells *et al.*, 2009).

Human muscles are heterogeneous in nature, comprising a mixture of different fibre types. The classification of fibre type can be based on contractile function, i.e. slow or fast twitch; metabolic characteristics, i.e. predominantly oxidative or glycolytic; any combination of these two i.e. slow oxidative, fast-oxidative or fast-glycolytic fibres; finally on biochemical properties such as on the expression of the myosin heavy chain (MHC) isoform present (i.e. MHC Type I, IIa, IIa/x or IIx). The MHC isoform present will dictate the speed that the fibre contracts and thereby the rate of cross bridging cycling and maximal shortening velocity of the fibre. In human muscle, fibres can be classified as type I (slow-twitch), type IIa (fast-twitch) and type IIx (fastest) (Bárány, 1967; Westerblad *et al.*, 2010). Rodents additionally express a type IIb fibre, but this is non-existent in human skeletal muscle (Allen *et al.*, 2008; Westerblad *et al.*, 2010).

Different fibre types have varying oxidative potential, calcium (Ca^{2+}) handling properties, metabolic profile contractile and relaxation speed (Allen *et al.*, 2008; Westerblad *et al.*, 2010)

and these properties are thought to enable varying contribution to different types of exercise. Exercise of high-intensity, short duration involves a heavy recruitment of type IIa and IIx fibres, in addition to the full recruitment of type I fibres, whilst a large proportion of type I fibres are recruited during longer duration events where a lesser force is produced, (Bottinelli *et al.*, 1999; Westerblad *et al.*, 2010)**.** Utilising changes in the PCr/Cr ratio it was determined that both type I and II fibres were recruited within 1 min of exercise at 75% of VO2 max, which corresponded to 38% of the maximal dynamic force; further, the same proportion of fibres remained activated during the 45 minute exercise period (Altenburg *et al.*, 2007).

2.1.1 Basic overview of excitation-contraction coupling

To enable skeletal muscle contraction, an AP must be propagated along the motor neuron to the neuromuscular junction, stimulating the release of acetylcholine (ACh). The release of ACh triggers a depolarisation of the motor end plate; generating an AP that propagates along the surface membrane and into the transverse tubules (t-tubules) of the muscle fibre. This signal is then conveyed to the sarcoplasmic reticulum (SR) via the voltage sensor dihydropyridine receptors (DHPR) (Payne & Delbono, 2004; Nielsen & Paoli, 2007). The DHPR transmits a voltage-mediated signal to open the ryanodine receptors (RyR) resulting in Ca^{2+} release from the SR into the cell cytoplasm (Dulhunty, 2006; Lamb, 2009). Calcium entering the cytoplasm binds to troponin causing tropomyosin to physically shift, uncovering the cross-bridge binding sites on the actin filament. The head of the myosin filament can then attach to actin, allowing cross-bridge cycling to begin (Payne & Delbone 2004, Nielsen & Paoli, 2007).

2.1.2 Skeletal muscle excitability

The AP propagation along the muscle fibre to commence EC coupling is dependent on the excitability of the muscle fibre which is largely regulated by the gradients and conductance's of the sodium (Na^+), potassium (K^+) and chloride (Cl^-) ions (Hodgkin & Horowicz, 1959). In

human skeletal muscle the resting membrane potential (E_m) has been calculated at -89 mV (Sjøgaard et al., 1985), but can range between -75 mV and -90 mV (McKenna *et al.*, 2008). In resting human skeletal muscle the interstitial [Na^+] was calculated to vary between 133-143 mM (Sjøgaard *et al.*, 1985) whilst intracellular values are quite low in comparison ranging from 6-13 mM (Cairns & Lindinger, 2008). In humans, resting plasma [K^+] is typically between 3.5-4.5 mmol.L^{-1}, whilst muscle interstitial [K^+] varies between 4.3 - 4.5 mmol.l^{-1} (Bergstrom *et al.*, 1971; Sjøgaard *et al.*, 1985; Green *et al.*, 2000; Juel *et al.*, 2000). When E_m is maintained between -75-90 mV, muscles are able to generate and propagate action potentials deep into the muscle fibre to allow excitation-contraction coupling (Nielsen & Clausen, 2000). The propagation of action potentials occurs within milliseconds, allowing prompt activation of muscle contractions (Clausen, 2010). Each action potential results in muscle cellular Na^+ influx and K^+ efflux (Sjøgaard, 1996). The rate of Na^+ entry into the muscle cell from an AP during an isometric contraction was reported as 1.9±0.2 and 20±4 nmol.g wet wt^{-1}·AP^{-1} in SOL and EDL, respectively (Clausen *et al.*, 2004), while the cellular K^+ efflux resulting from each AP has been reported to range between 1.7-2.0 µmol.kg $muscle^{-1}$ (Sjøgaard *et al.*, 1985; Hallén, 1996). Numerous studies utilising animals have demonstrated that during intense muscle contraction, the exchange of Na^+ and K^+ is sufficient enough to cause loss of excitability and to contribute to the onset of muscle fatigue and this is also believed to occur during exercise (Balog *et al.*, 1994; Nielsen & Clausen, 2000; Sejersted & Sjøgaard, 2000). A reduction in E_m can cause membrane depolarisation and cause inactivation the voltage Na^+ channels, which may reduce the frequency and amplitude of AP propagation (Rich & Pinter, 2003; Allen *et al.*, 2008). In addition, there is a large K^+ release from the muscle cell via Ca^{2+} and ATP dependent K^+ channels in addition to the voltage dependent K^+ gated channels involved in an action potential (Kristensen & Juel, 2009).

When rat EDL muscle was stimulated for 300 s at 5 Hz there was a significant net loss of K^+ from the muscle cell and this was associated by a smaller but significant uptake of Na^+ of which was enough to elicit a decrease in muscle excitability (Clausen, 2013a). Rat SOL and EDL muscles were exposed to an additional 7 mM of [K^+] which induced a decline in E_m to decrease from -76.5 ± 4.0 mV to ~ -60 mV in SOL and from -80.9 ± 3.3 mV to -63 mV in EDL (Cairns *et al.*, 1997). When E_m was reduced to -60 mV there was a 20% decrease in tetanic force production in both EDL and SOL, but when E_m was reduced to -60 to -55 mV the force decline was greater in EDL than SOL (Cairns *et al.*, 1997). Further increasing extracellular [K^+] by 7 mM in SOL muscle from 4 week old mice caused an 85% decrease in tetanic force, which was comparable with a reduction in the compound action potential (M-wave) area (Pedersen *et al.*, 2005). In human as a result of $[Na^+]_i$, $[K^+]_i$ and $[Na^+]_I$ $[K^+]_I$ shifts following knee extensor exercise there was a calculated 15 mV decrease in E_m (Sjøgaard, 1986).

The role of Cl^- conductance should briefly be discussed as during contraction Cl^- plays an important role in the maintenance of muscle excitability. Under resting conditions, Cl^- is known to be important for the maintenance of resting E_m (Hodgkin & Horowicz, 1959; Dutka *et al.*, 2008). During excitation where there is muscle cell depolarisation, additional to the K^+ efflux, there is an influx of Cl^- in to the muscle cell. This influx causes a repolarisation of the cell which increases K^+ uptake by the inward rectifier K^+ channels (Clausen, 2013a). During whole muscle experiments with rat SOL muscle where extracellular [K^+] was 11 mM, ensuring a loss of force was recovered by reducing Cl^- conductance (Pedersen *et al.*, 2005). In skinned muscle single fibres from rat EDL, force was reduced 4 times faster when the t-tubular Cl^- conductance and NKA activity were blocked together, compared to when than when NKA activity was blocked alone with t-tubular conductance present (Dutka *et al.*, 2008).

Despite the positive effects of Cl^- on E_m, there is still substantial evidence that Na^+ and K^+ exchange across the cell membrane during excitation impair muscle excitability which has been shown to lead to a reduction of force (Clausen, 2013a). In skeletal muscle the sodium-potassium pump (Na^+,K^+-ATPase or NKA) is a P-type ATPase and is responsible for the active transport of Na^+ and K^+ ions across the cell membrane against their concentration gradients and is expressed in a variety of tissues, including, but not limited to, skeletal and cardiac muscle, kidney, liver and brain (Blanco & Mercer, 1998). The NKA exchanges 3 Na^+ for 2 K^+ ions with the hydrolysis of one ATP molecule per cycle (Thompson & McDonough, 1996b). This process is critical in maintaining wide transarcolemmal Na^+ and K^+ concentration gradients, therefore preserving membrane potential (E_m) and therefore the NKA is the major focus of this thesis.

2.2 Skeletal Muscle Na^+,K^+-ATPase

2.2.1 Function and structure

Skeletal muscle has the largest pool of NKA in the body due to both a large tissue mass and relatively high NKA concentration (Clausen, 2003b). Declines in muscle NKA content or maladaptations to the NKA have been associated with some diseases and poor muscle function (Clausen, 2013b). In contrast, individuals with increased muscle NKA content have been shown to have improved muscle function and exercise performance (Section 2.4.3).

The NKA comprises alpha (α) and beta (β) subunits, which together constitute a functional αβ heterodimer (Green, 2004). The NKA α subunit (~100-112 kDa) contains binding sites for Na^+, K^+ and Mg^{2+} ions as well as phosphate and ATP and typically undergoes both phosphorylation and oxidation (Clausen, 2003b; Lingrel *et al.*, 2003; McKenna *et al.*, 2006). The β subunit (~35-55 kDa) is glycosylated and is necessary for the structural maturation of the α subunit, localisation of the NKA heterodimer to the sarcolemma and regulation of NKA activity

(Cougnon *et al.*, 2002). These NKA isoforms are differentially expressed throughout different tissues, which suggests the function and regulation of each isoform varies (McDonough *et al.*, 2002). Techniques used to study function of these isoforms include global knockout models, which are not successful in helping determine functions of each isoform, as a knockout of either of the three α isoforms leads to the death of rats and mice before or immediately following birth (Lingrel *et al.*, 2007). Research using isoform knockout models specific to skeletal muscle has however helped to establish the roles of the α_1 and α_2 isoforms (Radzyukevich *et al.*, 2013). Each subunit is expressed as several different isoforms, each coded to separate genes; including four α isoforms (α_1, α_2, α_3, α_4) and three β isoforms (β_1, β_2, β_3) (Blanco & Mercer 1998).

An accessory γ protein (8-14 kDa) also regulates the NKA, known as a FXYD protein.The γ subunit of the NKA co-immunoprecipitates with the α and β subunits and is a small, hydrophopic polypeptide (Blanco & Mercer, 1998). The FXYD protein is expressed as seven isoforms (FXYD1-7) and the main isoform expressed in skeletal muscle and associated with NKA regulation is the FXYD1, otherwise known as phospholemman (PLM) (Crambert *et al.*, 2002).

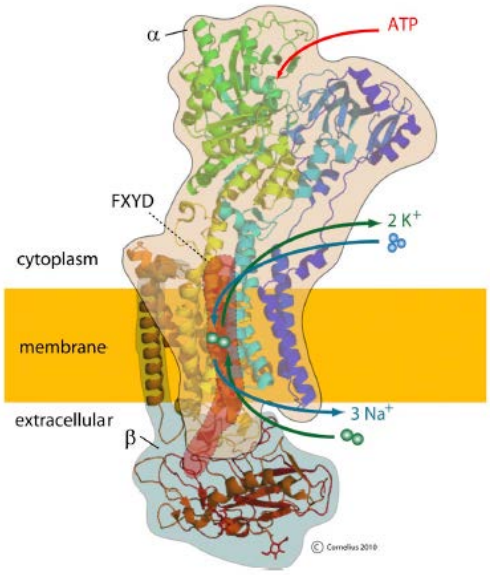


Figure 2.1 Molecular structure of Na^+, K^+-ATPase, drawn by Flemming Cornelius and published by Clausen (2013b)

2.2.2 Quantification of the NKA in human skeletal muscle

2.2.2.1 [^{3}H]ouabain binding

In skeletal muscle the most widely accepted method for quantification of the total number of functional NKA is through measurement of the [^{3}H]ouabain binding site content in muscle (Clausen, 2003a). The procedure is performed on small pieces of whole muscle samples (typically between 5-20 mg) and is based on the affinity binding of cardiac glycosides to the α subunit of the NKA, with a stoichiometry of 1:1 (Hansen, 1984). By incubation of muscle samples in tritiated ouabain and counting of β particles via liquid scintillation, it is possible to quantify the NKA in molar units, typically expressed as NKA content in pmol·g wet weight^{-1}(Hansen & Clausen, 1988). This can be contrasted to western blotting, which typically only gives measurement of relative abundance using an arbitrary unit.

In rat muscle the α_1 isoform makes up approximately 20% of the NKA α subunits; however its lower affinity to cardiac glycosides doesn't allow for α_1 to be detected using the standard [^{3}H]ouabain binding sites measurement (Hansen, 2001). Hence, in rat skeletal muscle, the α_2 is the only NKA α isoform which is measured in the [^{3}H]ouabain binding analysis. In contrast in humans, in skeletal muscle and other tissues, the three α isoforms have a similar ouabain affinity and so can be readily detected by [^{3}H]ouabain binding (Wang *et al.*, 2001; Clausen, 2013b). The [^{3}H]ouabain binding site content reported in human skeletal muscle is shown in Table 2. 1. However, the [^{3}H]ouabain binding assay does not identify which of the α isoforms may have been detected and furthermore no information can be obtained regarding abundance of the beta isoforms or FXYD1.

The use of rat skeletal muscle for quantifying NKA content can be used to gain a representation of NKA fibre type specificity, as the rat EDL and soleus muscles are comprised of predominately type II and type I fibres, respectively. Given the [^{3}H]ouabain binding procedure can only be performed in whole muscle pieces, measures conducted in human skeletal muscle are therefore unable to determine any possible fibre type differences due to the heterogeneous nature of human skeletal muscle from the vastus lateralis muscle.

In rat skeletal muscle it is unclear whether [^{3}H]ouabain binding site content is greater in a particular fibre-type. The [^{3}H]ouabain binding site content was higher in rat SOL and EDL by ~50% and in red vastus lateralis by ~70%, when compared to white vastus lateralis muscle, with no difference reported between SOL, EDL and red vastus lateralis (Chin & Green, 1993). The [^{3}H]ouabain binding site was 4 times greater in SOL compared with EDL in mouse muscle (Bray *et al.*, 1977). Whilst conversely there was [^{3}H]ouabain binding is between 23-50% higher in EDL compared to SOL (Clausen *et al.*, 1982; Kjeldsen *et al.*, 1984a; Everts & Clausen, 1992). The collation of data from numerous rat studies, all of which measure different muscles and use different aged rats makes a direct comparison between studies difficult.

2.2.2.2 Western blotting

Typically, western blotting is used to determine the relative abundance of an individual isoform compared to a housekeeping protein or the total protein per lane and is expressed in arbitrary units (a.u.). Additionally western blotting can be used to measure the phosphorylation state of proteins (Murphy *et al.*, 2006b) and specifically for the regulation of NKA, this is mainly FXYD1 phosphorlyation (Thomassen *et al.*, 2013). Many researchers have performed western blotting utilising separated fractions of homogenised skeletal muscle, to purify the sample analysed (Nielsen *et al.*, 2004; Mohr *et al.*, 2007; Bangsbo *et al.*, 2009). However, there are important adverse implications of this approach in analysing the

supernatant of a muscle homogenate, with the recovery of NKA was reported to only be between 0.2-8.9% (Hansen & Clausen, 1988). Therefore when determining NKA protein relative abundance, the use of a whole muscle homogenate (i.e. with no centrifugation) is a preferred method to recover all NKA molecules and gain the best representation of NKA isoforms in the muscle. However, the use of a whole muscle homogenate derived from human muscle biopsy samples doesn't allow for any fibre-type differences to be determined, since human skeletal muscle is heterogeneous with a relatively equal proportion of type I and II fibres.

Recently a new method has been developed which allows for the quantification of proteins within a single fibre segment (Murphy, 2011). This approach avoids two common problems with western blotting. Firstly, this allows a "whole muscle" sample to be analysed as the intact fibre segment encases the plasma membrane and all intracellular compartments of the cell, ensuring there is no loss of the NKA as would occur during any centrifugation procedures. Secondly, this method overcomes the problem of saturating a gel with a large amount of protein, which may potentially mask a research outcome following an intervention (Mollica *et al.*, 2009). Additionally, this technique allows for the detection of all NKA isoforms in different fibre-types. To date western blotting using single fibres has been used to explore the fibre-type expression of three of the six NKA isoforms ($\alpha_{1\text{-}2}$ and β_1) expressed in human skeletal muscle (Thomassen *et al.*, 2013). The determination of possible fibre-type specific expression of all six NKA isoforms expressed in human single skeletal muscle fibres and their adaptability to three interventions and ageing is a key focus of this thesis.

Table 2.1 Vastus lateralis muscle [3H]ouabain binding site content from muscle biopsy samples in healthy young adults aged between 18-35 years.

Study	[^{3}H]ouabain (pmol.g wet weight^{-1})
Norgaard *et al*., (1984)	278 ± 15
Dorup *et al.,* (1988)	258 ± 16
Kiltgaard *et al.,* (1989)	276 ± 19
Kjeldsen *et al.,* (1990)	308 ± 13
Benders *et al.* (1992)	360 ± 31
McKenna *et al.,* (1993)	333 ± 19
Green *et al.,* (1993)	339 ± 16
Madsen *et al.,* (1994)	307 ± 43
Schmidt *et al.* (1994)	223 ± 13
Gullestad *et al.* (1995)	258 ± 13
Ravn *et al.* (1997)	276 ± 11
Evertsen *et al.,* (1997)	343 ± 11
Haller *et al.* (1998)	281 ± 20
Green *et al.,* (1999)	289 ± 22
Green *et al.*(2000a)	348 ± 12
Medbo *et al.,* (2001)	356 ± 6
Fraser *et al.,* (2002)	311 ± 41
Leppik *et al.,* (2004)	317 ± 17
Nordsborg *et al.* (2005a)	312 ± 17
Aughey *et al.,* (2005)*	307 ± 41
Aughey *et al.* (2006)*	318 ± 37
Aughey *et al.,* (2007)*	355 ± 80
McKenna *et al.,* (2012)*	350 ± 108

Data is presented as mean ± SE unless otherwise stated. *SD. Range (Minimum-Maximum) 223- 355 pmol.g wet weight^{-1}. Mean from 23 studies presented is 308 pmol.g wet weight^{-1}.

2.2.3 Fibre-type specificity of the NKA isoforms in skeletal muscle

2.2.3.1 Alpha isoform fibre-type specificity

Based on experiments using isoform specific antibodies, the NKA α_1 isoform shows a similar abundance in oxidative compared to glycolytic muscle in the rat (Hundal *et al.*, 1993; Thompson & McDonough, 1996b; Ng *et al.*, 2003; Fowles *et al.*, 2004; Zhang *et al.*, 2006; Kristensen & Juel, 2010; Ingwersen *et al.*, 2011). The α_2 is similarly abundant in both oxidative and glycolytic muscles in the rat (Thompson & McDonough, 1996a; Fowles *et al.*, 2004; Kristensen & Juel, 2010; Ingwersen *et al.*, 2011). The fibre-type expression of these studies has been determined via western blotting, with the exception of Zhang *et al.*, (2006) who used immunohistochemistry techniques. The possible fibre-type specificity of α_3 has not yet been investigated in rodent skeletal muscle. Possible fibre-type specific expression of the NKA α isoforms in human skeletal muscle at a protein level has only been investigated in one study, which found the α_1 was similarly abundant in both type I and II fibres, whilst the α_2 was ~37% more abundant in Type II fibres (Thomassen *et al.*, 2013). To date the possible fibre-type difference of α_3 abundance has not yet been measured in human muscle.

2.2.3.2 Beta isoform fibre type specificity

In contrast to the α isoforms, studies in rat skeletal muscle have indicated distinct differences in β_1 expression between fibre types, with almost exclusive expression of β_1 in muscles rich in slow twitch fibres, whereas the β_2 is more abundant in fast twitch fibres (Hundal *et al.*, 1993; Thompson & McDonough, 1996b; Fowles *et al.*, 2004; Zhang *et al.*, 2006). The β_3 isoform in rat muscle was found to be similarly abundant in red and white gastrocnemius muscles (Ng *et al.*, 2003). In contrast to the rat, human skeletal muscle displayed a similar abundance of β_1 in type I and II fibres (Thomassen *et al.*, 2013). Neither the β_2 or β_3 isoforms have been measured in human single skeletal muscle fibres. This thesis will measure each of the α and β isoforms in human single muscle fibres, in both young adults and older populations.

2.2.3.3 FXYD1 fibre-type specificity

In rat, FXYD1 expression was higher in EDL compared to white gastrocnemius muscle when measured immunohistochemically (Reis *et al.*, 2005) but is expressed in both type I and II fibres (Bogaev *et al.*, 2001; Reis *et al.*, 2005; Rasmussen *et al.*, 2008; Galuska *et al.*, 2009). Only one study has investigated the FXYD1 total expression in human single fibres finding that FXYD1 abundance did not differ between type I and II fibres (Thomassen *et al.*, 2013).

2.2.4 Intracellular localisation of NKA isoforms in skeletal muscle

The α_1 isoform is ubiquitously expressed but in only skeletal muscle represents a small pool of the α subunits of approximately ~20%, whilst the α_2 is the most abundant comprising ~80% of the α subunits (Hansen, 2001; He *et al.*, 2001). The α_1 was shown to be expressed almost exclusively within the sarcolemma in rat EDL and SOL muscle, with a small amount also detected in the t-tubular system (Kristensen & Juel, 2010; Radzyukevich *et al.*, 2013). In human soleus muscle when analysed using immunohistochemistry, the α_1 expressed was also highly abundant in the sarcolemma (Hundal *et al.*, 1994).

In rat skeletal muscle, approximately ~50% of α_2 is located in the sarcolemma, with the remainder in the t-tubules (Hundal *et al.*, 1994; Lavoie *et al.*, 1995; Kristensen & Juel, 2010). And similarly in human soleus muscle, the α_2 was also found to be located within both the sarcolemma and t-tubular system (Hundal *et al.*, 1994). The α_3 was mainly present in the plasma membrane in human soleus, however the authors reported the use of a poor antibody which prevented a full investigation of the localisation of this isoform (Hundal *et al.*, 1994).

In rat skeletal muscle the β_1 and β_2 isoforms are expressed in both the sarcolemma and t-tubules (Hundal *et al.*, 1992; Lavoie *et al.*, 1996). In rat skeletal muscle the β isoform abundance was reported to be ~6 times greater in the cell membrane than in the t-tubules and compared to the α isoforms the β isoforms were approximately 5 times more abundant in the

cell membrane (Lavoie *et al.*, 1997). It was suggested a heterodimer with α:β ratio ≥ 1:2 had a higher catalytic activity than those with a 1:1 ratio of α:β subunits (Lavoie *et al.,* 1997). But there is a lack of understanding of how of a surplus of β isoforms contribute to increasing NKA activity or if they do not improve activity, what their function otherwise may be. In human soleus muscle, the NKA β_1 isoform was reported to be located mainly in the plasma membrane and a small amount within the intracellular fraction of muscle, but neither the β_2 nor β_3 isoforms have been investigated (Hundal *et al.*, 1994).

2.2.5 Specific functions of NKA isoforms

Understanding of the specific functions of the NKA isoforms is incomplete. In skeletal muscle the α_1 isoform plays appears to play a principal role in Na^+/K^+ transport and membrane excitability during basal conditions, since in skeletal muscle α_2 knockout mice, muscle excitability under basal conditions was not different to wild type mice (He *et al.*, 2001; Radzyukevich *et al.*, 2013).

The α_2 is believed to play role in maintenance of force production and muscle contractility. In skeletal muscle knockout α_2 mice and partial α_2 knockout mice (α_2 reduced by ~50%) marked decreases in treadmill running and muscle contractility were found compared to wild type mice (Lingrel *et al.*, 2003; Radzyukevich *et al.*, 2013). Further, in-vivo experiments from skeletal muscle α_2 knockout mice show a rapid decline in maximum twitch force and maximal tetanic force following repeated nerve stimulation (Radzyukevich *et al.*, 2013).

The α_3 is the least abundant of the alpha isoforms in rat skeletal muscle, with its role largely unknown (Blanco and Mercer 1998). It was earlier suggested that the α_3 isoform will be activated when there is membrane depolarization and large Na^+ and K^+ shift with both NKA α_1 and α_2 working at maximal in-vivo rates (Blanco & Mercer, 1998). Hence it was proposed that the α_3 is available as a “backup” isoform to help restore membrane potential (Blanco &

Mercer, 1998). It is known the α_3 isoform is largely expressed in neurons where it plays an important role in neurotransmission and in the maintenance of Na^+/K^+ gradients (Lingrel *et al.*, 2007; Bøttger *et al.*, 2011). Mice that are α_3 haplo-insufficient did not appear to be physically limited but were slower in learning tasks (Lingrel *et al.*, 2007). Furthermore zebra fish which were deficient of α_3 displayed abnormal brain ventricle dilation and abnormal mobility, likely to be caused by ion imbalances (Doganli *et al.*, 2013). Nothing is known about the NKA α_3 function in skeletal muscle.

The β isoform performs a regulatory role in the NKA and thus contributes to NKA activity. However, unlike the α isoforms, no β isoform knockout studies have been conducted, and thus the individual roles of the β isoforms are unknown. Under basal conditions, the β_1 has a higher affinity to Na^+ and lower affinity for K^+ compared to β_2, independent of the α isoform paired with (Crambert *et al.*, 2000).

2.2.4.3 Phospholemman (FXYD1/PLM)

The FXYD1 mainly associates with the α_1 and α_2 isoforms in skeletal muscle (Reis *et al.*, 2005; Geering, 2006; Rasmussen *et al.*, 2008). PLM in the unphosphorylated state binds to the α subunits and reduces Na^+ affinity (Galuska *et al.*, 2009) but when phosphorylated increases Na^+ affinity (Bibert *et al.*, 2008). Phospholemman acts as a main substrate for protein kinase A and C phosphorylation in skeletal muscle (Geering *et al.*, 2003) and appears that PLM is necessary for maximal activation of the NKA (Reis *et al.*, 2005).

2.2.6 NKA acute regulation

Multiple mechanisms exist to increase NKA activity in skeletal muscle (Clausen, 2013b) and are listed in Table 2.2. Given that is thesis does not investigate acute regulation of the NKA these will only be briefly discussed here, but further detail can be found in excellent reviews (Clausen, 2003b, 2013b).

Table 2.2 Factors which stimulate skeletal muscle NKA activity.

Stimulating factor	**Mechanism behind increased NKA activity**
Epinephrine	Stimulate cAMP which triggers activation of PKA thereby increase affinity of NKA forNa^+
β_2 agonists (Salbutamol, Isoproterenol)	Stimulate cAMP generation
Calcitonins	Stimulate cAMP generation
Amylin	Stimulate cAMP generation
Calcitonin gene related peptide (CGRP)	Stimulate cAMP generation
Theophylline	Degrades cAMP- leading to increased intracellular accumulation of cAMP
Monensin	Increases Na^+ concentration
Insulin/IGF-1	Increases Na^+ affinity of NKA Possible translocation role
Veratridine	Increases Na^+ influx per action potential
Excitation	Increases Na^+ influx
ATP/ADP ratio	Activation of purinergic receptors

Modified from Clausen (2013b)

2.2.6.1 Intracellular Na^+

The most common cause of rapid NKA activation is through excitation-induced rise in intracellular [Na^+] (Everts & Clausen, 1992). At rest, it has been proposed that the NKA activity is only at 2-6% of its maximal capacity (Clausen *et al.*, 1987) whereas in isolated

muscle loading muscle with Na^+ at 30 ºC, NKA could reach up to 90% of maximal theoretical activity (Clausen *et al.*, 1987). Stimulation of SOL muscle at 60 Hz for 10 s increased extracellular [Na^+] content by 58%, following recovery from stimulation $[Na^+]_i$ was returned to baseline within 2 minutes and was followed by an undershoot in $[Na^+]_i$ showing increased activity of the NKA, this was suggested to be at 15-fold times greater than rest (Everts & Clausen, 1994). This also demonstrates that increases in $[Na^+]_i$ is not required for elevation of NKA activity.

2.2.6.2 Phosphorylation of FXYD1

Phospholemman can be phosphorylated in skeletal muscle by both insulin and exercise (Crambert *et al.*, 2002; Benziane *et al.*, 2011). The phosphorylation of FXYD1 results from activation of protein kinases A (PKA) and C (PKC) (Bibert *et al.*, 2008; Thomassen *et al.*, 2011). Depending on the pathway used to phosphorylate FXYD1, different NKA isoforms may be impacted differently. In Xenopus oocytes, phosphorylation via protein kinase A had no effect on the K^+ affinity of α_1/β_1isoforms. Activation by PKA phosphorylation alone of PLM influences the apparent Na^+ affinity of the Na^+, K^+-ATPase α_1/β_1 and α_2/β_1 heterodimers but did not affect maximum NKA activity (Bibert *et al.*, 2008).

Contraction-induced FXYD1 phosphorylation occurs at multiple sites in human skeletal muscle including Ser^{63}, Ser^{68} and $Thur^{69}$ (Thomassen *et al.*, 2011). Phosphorylation was more pronounced after 20 minutes of moderate intensity exercise than after 30 s of high-intensity exercise (Thomassen *et al.*, 2011). It may be that intracellular signalling results in full phosphorylation of protein targets, optimal pump activity and maximum cellular K^+ uptake (Thomassen *et al.*, 2011). It has been suggested that PLM phosphorylation during exercise is dependent on contraction-induced signalling events. One of the possible upstream regulators is Ca^{2+} calmodulin-dependent protein kinase (CaMKII) and it has been suggested that PLM contains a phosphorylation site for CaMKII (Benziane *et al.*, 2011).

2.2.7 Chronic adaptability of skeletal muscle NKA

2.2.7.1 Hormonal regulation

2.2.7.2 Thyroid hormone

Of the multiple hormones which play roles in the chronic regulation of the skeletal muscle NKA, thyroid hormones are the most potent in influencing NKA expression and in upregulating NKA content and activity (Asano *et al.*, 1976; Clausen, 2013b). Hyperthyroid rats showed increased skeletal muscle NKA content in EDL (2.6 fold greater), gastrocnemius (5.1 fold greater) and SOL (9.8 fold greater) compared to hypothyroid rats (Kjeldsen *et al.*, 1986). They were not compared to wild-type rats, furthermore, this was more specific to type I than type II fibres (Kjeldsen *et al.*, 1986). In human muscle, NKA content measured by [^{3}H]ouabain binding was decreased in patients with hypothyroidism by 50% compared to euthyroid patients, whilst those with hyperthyroidism had a 68% increase of NKA content of compared to euthyroid controls (Kjeldsen *et al.*, 1984c). The NKA isoform abundance has not been measured in humans with either hypothyroidism or hyperthyroidism.

2.2.7.3 Growth Hormone

Injection of growth hormone (GH) in K^+ deficient rats did not upregulate the skeletal muscle NKA content; however, rodents with a normal electrolyte status infused with GH did exhibit upregulated NKA content (Dørup *et al.*, 1992). There was also an increase in IGF-I with GH and it was suggested that these two hormones may play a role in NKA synthesis (Dørup *et al.*, 1992).

2.2.7.4 Corticosteroids

Corticosteroids have long-term effects on NKA regulation inducing both mRNA and protein expression (Therien & Blostein, 2000). Dexamethasone infusion in rats for 7-14 days increased NKA content in SOL, EDL, gastrocnemius and diaphragm by 22-48% without

altering intramuscular Na^+/K^+ contents (Dørup & Clausen, 1997; Thompson *et al.*, 2001). Infusion of dexamethasone for two weeks significantly increased the α_1 isoform abundance in diaphragm muscle only (Thompson *et al.*, 2001). The NKA α_2 isoform was increased by 53–78% compared to controls in all muscles analysed, whilst in EDL and gastrocnemius the β_1 was also upregulated following dexamethasone (Thompson *et al.*, 2001). In humans, 5 days of dexamethasone oral supplementation (2 mg per day) increased vastus lateralis muscle [^{3}H]ouabain binding in addition to NKA isoform α_1 and α_2 protein abundance by ~17% and β_1 and β_2 protein abundance by ~6-8% (Nordsborg *et al.*, 2005a).

Conversely, rats that were infused with aldosterone for 7 days had a reduction in the total NKA content (Dorup, 1996).

2.2.7.5 K^+ depletion and supplementation

Animals that underwent dietary K^+ depletion had a reduction in skeletal muscle NKA content by as much as 76% (Nørgaard *et al*. 1981, Kjeldsen *et al*. 1984b). Rats deprived of dietary K^+ in their diet for 10 days showed reductions in α_2 protein abundance of 30% in the diaphragm, 60% in soleus and greater than 90% in white gastrocnemius (McDonough *et al.*, 2002). Additionally, the β_1 was decreased by 20% in soleus whereas β_2 decreased by 75% in white gastrocnemius. The reduction in NKA with K^+ depletion may be caused by decreased synthesis rate and/or increased degradation rate of the subunits (McDonough *et al.*, 2002).

Potassium supplementation in rats had the opposite effect, with an increased [^{3}H]ouabain binding evident after 7 days of K^+ supplementation until 2 weeks where NKA content was 68% higher in gastrocnemius muscle (Bundgaard *et al.*, 1997). The marked reduction in NKA in rats following K^+ deprivation was restored to control levels within 6 days of K^+ repletion (Kjeldsen *et al.*, 1984b). Interestingly, intramuscular K^+ was restored within 24 h and supports claims from other studies that the total NKA content is related to intramuscular K^+ content (Kjeldsen *et al.*, 1984b).

2.3 Exercise training overview

Recently high-intensity training (HIT) has become a common training modality in research. High-intensity training consists of multiple bouts of exercise (4-10), lasting between 10 seconds up to 4 min duration, of which, completed at a high-intensity at ≥ 80% of VO_2 peak. These efforts are interspersed with recovery between each bout of exercise, often multiple sets of these efforts are completed in a given training session (Gibala & McGee, 2008; Iaia & Bangsbo, 2010; Gibala *et al.*, 2012). The potential applications of high-intensity training are vast, given that the most commonly reported barrier preventing the general population from exercise is lack of time (Stutts, 2002; Kimm *et al.*, 2006). The use of intermittent exercise provides an alternative to continuous training, whilst still providing ample health and fitness benefits (Wisloff *et al.*, 2007; Helge *et al.*, 2010; Krustrup *et al.*, 2010; Gunnarsson & Bangsbo, 2012; Krustrup *et al.*, 2013; Barker *et al.*, 2014; Lunt *et al.*, 2014). Further, adaptations gained from HIT are comparable to and often greater than those training effects induced by moderate-intensity long duration training (Harmer *et al.*, 2006; Wisloff *et al.*, 2007; Trapp *et al.*, 2008; Gillen *et al.*, 2013; Tjønna *et al.*, 2013; Esfandiari *et al.*, 2014).

Repeat-sprint exercise (RSE) comprises multiple (4-6) high-intensity bursts, each lasting between 2-6 s, interspersed by a brief recovery period (Spencer *et al.*, 2005) and are typically comparable with efforts produced during intermittent team sports, such as Australian football, soccer, rugby and hockey (Aughey, 2010; Jennings *et al.*, 2012; Varley *et al.*, 2013). RSE training has only sparsely been used as a training modality in research, which is surprising given that repeat sprint ability has been strongly correlated to high-intensity running in soccer (Rampinini *et al.*, 2007), while multi-set RSE training (comprising three sets of 5x4 second sprints) improves acceleration, Yo-Yo Intermittent Recovery Test 1 performance (Serpiello *et al.*, 2011) and upregulated proteins associated with mitochondrial biogenesis in skeletal

muscle (Serpiello *et al.*, 2012). No research has yet investigated the effects of RSE training on NKA isoform abundance and this is investigated in Chapter 3.

2.3.1 Training and skeletal muscle NKA

Exercise comprising long duration of a moderate intensity, has been shown to be effective at increasing NKA content, isoform abundance and improving exercise performance (Table 2.3).

Continuous cycling for 2 hrs at 65% VO_2 max for only six days increased [^{3}H]ouabain binding by 13.6% (Green *et al.*, 1993). When the same training was conducted 3 d per week over an 11 week period [^{3}H]ouabain binding was increased by 22%; this increase was observed after the first 3 weeks, after which there were no further increases (Green *et al.*, 1995). Sprint training comprising 4 x 30 s bouts, 3 days per week sprints, with gradual increases in the number of sprints completed and reducing the recovery over 7 wks increased muscle [^{3}H]ouabain binding by 16% (McKenna *et al.*, 1993). No changes in muscle [^{3}H]ouabain binding or NKA isoform abundance were seen in elite cyclists completing additional interval training involving 8 x 5 min at 80% of peak power (Aughey *et al.*, 2007). Speed endurance training comprising of 8 x 30s efforts at 130% of VO_2 max increased the relative abundance of the α_1 and β_1 isoform (Mohr *et al.*, 2007). Conversely, 12 x 30s runs at 90-95% peak running speed over 4 weeks increased α_2 isoform abundance only, with no changes in the NKA α_1 and β_1 isoforms (Bangsbo *et al.*, 2009). Further, 10 days of training involving cycling at 75% VO_2 peak for 45 -90 minutes and HIT comprising 6x5 min at ~90-100% of VO_2 peak increased the relative abundance of NKA α_1 (113%), α_2 (49%) and β_1 (27%) (Benziane *et al.*, 2011). These studies demonstrate the NKA adaptability following differing training modalities consisting of training with a reduced volume and high intensity and more traditional long duration training programs. In this thesis, two studies focus on

NKA adaptation in skeletal muscle following intensified training, using RSE training in healthy young adults in Chapter 3 and chapter 6 HIT in the elderly in Chapter 5.

Table 2.3 Exercise performance and skeletal muscle NKA adaptations to intense exercise training in healthy young humans

Reference	n	Pre-train VO_2 peak ($ml.kg.min^{-1}$)	Age (yr)	Training Type /frequency	Training session details	Training duration (wk)	Performance measure change	Isoform abundance	[^{3}H] Ouabain binding (%)
1	15	NR	20	Military Moderate Physical Training	NR	10	↑ 7% distance during 12 min run test	NM	n.c following training
2	6	51.1	18.8	x3 p/wk Wk1- 4x30s bouts Wks 4-7 10x30s sprint bouts	30s maximal cycle sprints	7	↑ 11% work output	NM	↑ 16%
3 (mean±SE)	9	47.5	19.7	Continuous	65% VO_2 max 2 hours	6 days	↑ 6.5% $VO2_{max}$	NM	↑ 13.6%
4	39		30	x3 p/wk high-intensity x1-3 p/wk low intensity run	93% of HR max Low-intensity run < 60% HR max	6	↑ 5%$VO_{2\ max}$	NM	↑ 15%
5	16	45	21.4 19.9	x3 p/wk	Submax group ~68% $VO_{2\ peak}$ ~ 2 hours Resistance Group 3 sets 8-10 reps	11 12	↑$VO_{2\ peak}$ In submaximal group	NM	↑22% submax group ↑16% Resistance group

6	20	66.6	18	7 days p/wk	Skiing, Running MI group- 86% of training at 60-70% $VO_{2\ max}$ HI group 83% of training 80-90% $VO_{2\ max}$	5 month	↑ Distance in 20 min treadmill test	NM	↑16% in both groups No difference between groups
7	23	58	27	x1 p/wk (n=7) x2 p/wk (n=7) x3 p/wk (n=7)	Strength training	3 month	↑ max strength all groups	NM	n.c x1 p/wk ↑ x2 p/wk ↑x3 p/wk
8 (mean±SE)	6	50.2	25.3	wk 1-2, x3 p/wk wk 3-4, x4 p/wk wk 5-7, x5 p/wk	Intermittent knee extensor exercise- Single leg, 15 work intervals ~150% of thigh VO2 max.	7	↑16% power output ↑Time to fatigue 27%	↑α_1 29.0 ± 8.4% ↑ α_2 15.1 ± 2.7% n.c β_1	NM
9	12	4.98 ($L.min^{-1}$)	31	Wk 1- x3 p/wk Wk 2- x2 p/wk Wk 3 x2 p/wk	Interval training 8x5min at 80% peak power output	3	↑ Peak power output 3%	n.c- $\alpha_1, \alpha_2, \alpha_3$ n.c- $\beta_1, \beta_2, \beta_3$	n.c following training
10	13	Sprint train group (ST) 50.2 speed endurance training (SET) group 49.0	26.7 24.6	Wk 1-2, x3p/wk Wk 2-5, x4 p/wk Wk 6-8 x 5p/wk Final week- 6 times p/wk.	Sprint training 15 x 6s 95% max running speed Speed endurance training 8x30s 130% VO_2 max	8	↑10%Yo-Yo IR2 ST & 30% SET ↑~18% time to exhaustion (SET) ↓~5.8%- 50m sprint (ST) ↓ 30m time (both)	n.c- α_1 in either group ↑ α_2 speed endurance training only (68±26%) ↑ β_1 both	NM

11	12	44.8	19.2	Continuous	~60% VO_2 max 2hrs	3 d	NM	↑ α_1 & α_2 ↑ β_1	↑ 12%
12	15	55.8	33.4	Sprint training (ST) 3-4 sessions per week CON 3-5 days per week	ST. 8-12 x30s runs at 90-95% max running speed. CON- normal training (9-12km, 45-60 min/day	4	↑ Yo-Yo IR2 19.0%- ST	↑ α_1 ~29% (ST) n.c α_2 n.c β_1	NM
13 *(mean±SE)*	17	63.0	34.8	SET a) 2-3 p/wk b) 1 p/wk c)1-2 p/wk	SET sessions a) 30s bouts at ~95% of max running speed. b) 4x4 min at >85% max HR c) <75% max HR or 75-85% max HR	6-9	n.c $VO_{2\ max}$ ↓ 3km run performance ↑ mean speed during 3km run	n.c α_1 ↑ α_2 68% (SET) n.c β_1	NM
14 *(Mean±SE)*	18	55.0	23.4	5 sessions of aerobic high-intensity (AHI) 5 sessions SET	AHI 8x2 min-4 vs.4 small sided soccer drills. 1 min rec SET 10-12 x 25-30s	2	↑ performance in 4th, 6th and 10th sprint in repeat sprint test ↓Total sprint time	n.c α_1 ↑ α_2 14.5 ± 4.9% n.c β_1	NM
15 *(mean±SE)*	18	60.6	23.9	x1 per week + regular soccer commitments	6-9 intervals at 90-95% maximal intensity		↓ O_2 consumption at 10 $km.h^{-1}$ ↑ Yo-Yo IR2 11%	n.c α_1 n.c α_2 ↓ β_1 13%	NM

16 *(mean±SE)*	18	52.2	33.8	3-4 x 5 minute running. Each 5 min consisting of 1 min intervals at <30%, <60% and 90-100% of running speed	7	↑10-20-30 performance by 6% ↑ $VO_{2\ max}$ 4%	n.c α_1 n.c α_2 n.c β_1	NM

Reference list; 1 Kjeldsen *et al.*, 1990 *Int J Sports Med*; 2. McKenna *et al*., 1993 *J. Appl. Physiol*; 3. Green *et al.*, 1993 *Am. J.Physiol*; 4. Madsen *et al.*, 1994, *Acta Physiol Scand* 5. Green *et al.*, 1999 *Acta Physiol Scand*; 6 Evertsen *et al.*, 1997 *Am. J. Physiol* ; 7 Medbo *et al.*, 2001*Eur J Appl Physiol*; 8 Nielsen *et al.*, 2003 *J. Physiol*; 9. Aughey *et al.*, 2007 *J. Appl Physiol*; 10. Mohr *et al.*, 2007*Am J Physiol Regul Inter Comp Physiol*; 11. Green *et al.*, 2008, *Am J Physiol Endocrinol Metab*; 12. Iaia *et al.*, 2008, *Am J Physiol regul Comp Physiol* 13. Bangsbo *et al.*, 2009 *J. Appl Physiol; 14.* Thomassen *et al.*, 2010, *J Appl. Physiol*; 15. Gunnarsson *et al.*, 2012,*Med. Sci. Sports & Exercise*; 16. Gunnarsson *et al.*, *2012*, *J. Appl. Physiol.*

All data presented as mean ± SD unless otherwise stated. SE= Standard error of the mean. NM= not measured. * Significantly increased from pre-train ($P<0.05$), ‡ significantly decreased from pre-train ($P< 0.05$) n.c= no change

2.3.2 Exercise Training and PLM

Training incorporating both continuous exercise at ~75% VO_2 peak for 45-60 min and high-intensity interval training for 10 days had no effect on skeletal muscle FXYD1 even though NKA α_1, α_2 and β_1 isoform abundances were increased (Benziane *et al.*, 2011). However 14 week aerobic training increased FXYD1 by 154% in EDL, 150% in RG and 152% in WG muscles of old rats (Reis *et al.*, 2005). Discrepancies exist between the reported effects of exercise training and FXYD1 phosphorylation. Two weeks of intense soccer training increasing FXYD1 phosphorylation on site Ser^{68} (Thomassen *et al.*, 2010), whereas Benziane *et al.*, (2011) claimed FXYD1 is less likely to be involved in long-term skeletal muscle adaptability given that training did not modulate the FXYD1 phosphorylation response on Ser^{63}, Ser^{68}, or Thr^{69}. This was in addition to no change in FXYD1 protein abundance following training (Benziane *et al.*, 2011).

2.4 Inactivity

Inactivity has important adverse implications for health and muscle function, including in athletes recovering from injury, general population, patients with chronic disease and the aged. Different models of inactivity used to study these effects have unique advantages but also limitations. Bed rest studies are both highly expensive and difficult to recruit participants, whilst utilising a plaster cast on a limb to induce immobilization has major limitations, as the fixed joint angles by various protocols can influence muscle tone and cell metabolism and compromise results of studies (Booth, 1977). The recruitment of volunteer participants into these studies is highly difficult as they cause a major intrusion and disruption of a regular lifestyle. For these very important and practical reasons this thesis investigated inactivity utilising the unilateral lower limb suspension model.

2.4.1 Introduction to unilateral lower leg suspension model

Unilateral lower leg suspension (ULLS) is a model of inactivity designed to examine the effects of inactivity and unloading of one limb (Berg *et al.*, 1991) and requires participants to walk on one leg, typically with the other leg hanging freely and supported by the assistance of crutches. The use of ULLS allows the unloaded limb to move through a variety of joint angles (Hackney & Ploutz-Snyder, 2012). Two published forms of ULLS have been employed; the first employed a strap to support the unloaded leg to avoid unintentional weight bearing activity, however this approach was associated with greater risk of participants developing deep vein thrombosis (Berg *et al.*, 1991). A revised protocol was then developed to enhance participants safety that allows the unloaded leg to hang freely, by using an elevated platform shoe on the weight bearing leg (Figure 2.2) (Tesch *et al.*, 2004; Hackney & Ploutz-Snyder, 2012).

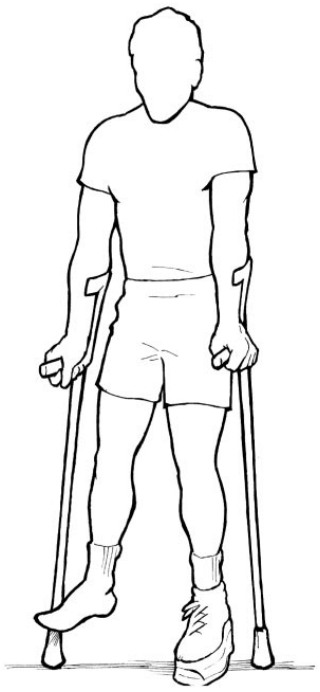


Figure 2.2 Unilateral lower limb suspension. From Tesch *et al.*, (2004).

2.4.2 Functional and structural effects on skeletal muscle following ULLS

The use of ULLS results in functional declines in knee extensor muscle isometric force that are similar to bed rest studies, reported at approximately 0.73% decline per day (Hackney & Ploutz-Snyder, 2012). Peak knee extensor muscle torque was reduced by 19% following four weeks of ULLS and was restored after 7 weeks of undirected recovery (Berg *et al.*, 1991). Maximum knee extensor torque has also been reduced following even 10 days of ULLS (Berg & Tesch, 1996). When specific force was measured in soleus muscle, ULLS has been suggested to induce a larger loss of specific force than in bed rest models (Widrick *et al.*, 2002). Few studies have investigated the effects of unloading on muscular endurance, but total work capacity of the knee extensors during isokinetic knee extension exercise was reduced by 13% after 21 days of ULLS and by 24% after 30 days (Schulze *et al.*, 2002; Cook *et al.*, 2010).

Following four weeks of ULLS there was no change in body mass; however, knee extensor muscle mass measured by CT scan was decreased by 7% (Berg *et al.*, 1991; Schulze *et al.*, 2002). An 8% decrease in quadriceps cross-sectional area occurred following lower limb suspension for 16 days measured using magnetic resonance imaging (Adams *et al.*, 1994). No change in muscle fibre-type distribution has been found in human muscle following inactivity (Berg *et al.*, 1993; Adams *et al.*, 1994).

2.4.3 Molecular adaptations to skeletal muscle NKA following inactivity

Whilst the detrimental effects of unloading on muscle function and size have been well described after ULLS, little is known about the molecular adaptation to proteins involved in muscle excitation-contraction coupling, particularly in the NKA. The effects of inactivity/injury on NKA have been studied using other models. In ovine skeletal muscle, [^{3}H]ouabain binding was reduced by 39% after cast immobilisation for 9 weeks (Jebens *et al.*, 1995). In human shoulder impingement syndrome, which immobilised the deltoid muscle,

deltoid NKA content was reduced by 27% compared to the uninjured shoulder (Leivseth & Reikeras, 1994). People with complete spinal cord injury had ~less than half skeletal muscle NKA content than able-bodied controls (Ditor *et al.*, 2004; Boon *et al.*, 2012). A recent study has investigated NKA isoform abundance in muscle homogenates following complete spinal cord injury and found reductions in both the NKA α_1 and α_2 isoforms by 75% and 51%, respectively, whilst β_1 was reduced by 38% when compared to able bodied controls (Boon *et al.*, 2012). No studies have investigated the effects of disuse per se, without potentially confounding effects of injury and medications on NKA content and isoform abundance. Whether inactivity affects the abundance of NKA isoforms in a fibre type specific manner in humans is also yet to be determined and these are investigated using ULLS in Chapter 6.

2.5 Ageing, skeletal muscle NKA adaptability and exercise training

2.5.1 Exercise performance in the elderly

Ageing results in an unavoidable but gradual decline in muscle strength and function, coinciding with a decline in muscle mass, referred to as sarcopenia (Porter *et al.*, 1995). Additional to sarcopenia are neuromuscular maladaptations which include altered muscle fibre innervation (Payne & Delbono, 2004) and loss of motor units (Aagaard *et al.*, 2010). Combined, these contribute to the reduced capacity of older adults to perform activities of daily living and reduction in quality of life (Evans, 2010). Aerobic power, a key indicator of mortality in clinical populations (Blair *et al.*, 1996; Smart, 2013; Weston *et al.*, 2013) is also decreased in older adults compared to young (Proctor & Joyner, 1997). Numerous mechanisms contribute to the decline in aerobic power and exercise capability in the elderly, including reduced maximum cardiac output, lower HR and arteriovenous oxygen difference at maximal exercise, along with reduced muscle mass and reduction in the number of type II fibres (Ogawa *et al.*, 1992; Porter *et al.*, 1995; Mishra & Misra, 2003; Kwak, 2013). The next

section briefly discusses the adaptation of skeletal muscle NKA in ageing and impact of the use of differing aerobic exercise modalities in the elderly on NKA.

2.5.2 Skeletal muscle adaptations to ageing

Ageing skeletal muscle undergoes fibre atrophy, with type IIa/x fibres more affected then type I (Lexell *et al.*, 1988), likely due to reduced myofibril protein synthesis compared to young adults (Welle *et al.*, 1995). Other skeletal muscle proteins are also adversely effected by ageing, with decreases in GAPDH in glycolytic rat fibres (Lowe *et al.*, 2000) and monocarboxylate transporter (MCT) 1 in SOL muscle and MCT4 in EDL in old compared young rats (Masuda *et al.*, 2009). Important ion-regulatory proteins also decline with ageing including decreases in dihydropyridine receptors and ryanodine receptors in aged rat SOL muscle compared to young (Payne & Delbono, 2004). In humans muscle mitochondrial function is declined with ageing (Johnson *et al.*, 2013). The NKA has been only sparsely investigated in ageing human muscle and is covered in the final part of this literature review.

2.5.3 Skeletal muscle NKA content in age

In rats, [^{3}H]ouabain binding sites increased from birth up until 1 year of age followed by a 50-70% decline over the following 2-20 months (Kjeldsen *et al.*, 1984a). There was also a 58% decrease in [^{3}H]ouabain binding sites in the SOL muscle from rats aged between 28 and 85 days (Clausen *et al.*, 1982). Conversely, there was no difference in [^{3}H]ouabain binding in in either EDL or SOL muscles from 3 to 14-16 month old rats (Abdel-Azia *et al.*, 1985).

In humans skeletal muscle [^{3}H]ouabain binding tended to be 14% (non-significant) lower in 28 vs. 68 years of age (Klitgaard & Clausen, 1989). Similarly no difference was detected in [^{3}H]ouabain binding between older adults with a mean of 66.8 years and young adults with a mean age of 23.9 years (McKenna *et al.*, 2012). The observation of no significant differences in NKA content between young and older humans may be explained by numerous

possibilities. Firstly, it may be possible that the elderly participants tested previously were simply not old enough for a decline to be evident. In support of this, older adults aged between 69-81 years had a 25.5% lower [^{3}H] ouabain binding compared to those aged 55-68 years (Perry *et al.*, 2013). The lack of change seen with ageing in previous studies might also be explained by smaller muscle fibres and increased relative membrane density in the elderly. In porcine muscle, smaller muscle fibres had significantly greater membrane surface area per unit volume, which was related to [^{3}H]ouabain binding sites (Harrison *et al.*, 1994). Despite no change in NKA content, there was a 24% decrease in α_2 isoform protein in older adults (McKenna *et al.*, 2012). The α_2 is the most abundant NKA isoform in skeletal muscle (~85%) (He *et al.*, 2001) and in human was reported to be more abundant in type II fibres by ~37% (Thomassen *et al.*, 2013). During ageing, type II fibres are more affected by atrophy and this may contribute to the decrease of α_2 in ageing. A mixed fibre muscle sample analysed during [^{3}H]ouabain binding may confound a total decrease in [^{3}H]ouabain binding, but currently there is no way to measure [^{3}H]ouabain binding in single fibres of human skeletal muscle. Despite these potential mechanisms, the study conducted by McKenna *et al.*, (2012) did not see an upregulation of other α isoforms observed in ageing that had been noticed in other studies. Often the upregulation of other isoforms is seen with a decreased α_2 and could explain no change in [^{3}H]ouabain binding (McKenna *et al.*, 2012). These studies to date have been conducted using whole muscle homogenate and are unrepresentative of changes occurring on the single fibre level and some results may be confounded by the use of whole muscle.

2.5.4 Adaptations of NKA isoforms to ageing

Alterations to the NKA isoforms during ageing in rats are inconsistent (Sun *et al.*, 1999; Ng *et al.*, 2003; Zhang *et al.*, 2006). The use of different ages of rats studied and different muscle analysis techniques makes direct comparisons between studies difficult; nonetheless, these

studies collectively establish that in rat skeletal muscle, the NKA undergoes adaptations with ageing. An advantage of rodent studies is they allow insight into fibre type specificity of isoforms, which is currently lacking in human skeletal muscle research due to the heterogeneous composition of human skeletal muscle. A summary of studies in rodent investigating isoform abundance with ageing studies can be found in Table 2.5. There appears to be a greater abundance of α_1 and β_1 isoforms with ageing (Ng *et al.*, 2003). This may result in more $\alpha_1\beta_1$ type NKA heterodimers in aged skeletal muscle. As $\alpha_1\beta_1$ have a higher Na^+ affinity in resting muscle (Kristensen & Juel, 2010), an increased α_1 and β_1 with ageing may influence Na^+/K^+ regulation across the plasma membrane during resting conditions.

A recent study investigated NKA isoform abundance in human muscle and found a 24% decrease in α_2 and 23% decrease in β_3 isoform abundances in older adults (McKenna *et al.*, 2012). This analysis was conducted in whole muscle homogenates, so any possible fibre type differences for these isoforms could not be determined. Furthermore additional changes that were seen in rat may be concealed by the use of whole muscle homogenate. To determine whether fibre-specific changes in NKA isoforms occur with ageing, analyses on the single fibre level were conducted in Chapter 4.

Table 2.4 Skeletal muscle NKA isoform abundance changes with ageing in rats

Study	n	Isoform	Age Young (months)	Age Old (months)	EDL	SOL	RG	WG
1	not reported	α_1	6	30	NM	NM	↑*	↑*
		α_2			NM	NM	↓*	↓*
		β_1			NM	NM	↑*	↑*
		β_2			NM	NM	↓*	↓*
		β_3			NM	NM	↑*	↑*
2	12-15 Per group	α_1	16	29	NS	NM	↑	↑
		α_2			NS	NM	NS	NS
		β_1			NS	NM	NS	NS
		β_2			↓	-	↓	↓
		β_3			-	-	↑	↑

Study	n	Isoform	Age Young (months)	Middle Age (months)	Aged Old (Months)	SOL	RG	WG
3#	6	α_1	6	18	30	↑ compared to 6month only	↑ compared to 6month only	↑ Compared to both 6 & 18 month
		α_2				↓ at 18 months only	↓ at18 & 30 months	↓ 18 & 30 months
		β_1				NM	↑ compared to both 6 & 18 month	↑ at 30 months only
		β_2				NM	↓compared to 6 month only	↓18 & 30 months

1 Zhang *et al.,* 2006, 2. Ng *et al.,* 2003 3. Sun *et al.,* 1999. # EDL not measured. NS not significantly different between old and young; NM; not measured↑ old > young $p<0.05$; ↓ old < young $p<0.05$; ↑* increase but no statistical analysis or p value reported; ↓* decrease but no statistical analysis or p value reported.

2.5.5 Isoform adaptations to exercise training in aged rats

Only one study to date has investigated changes in NKA isoform abundance in aged rat muscle with exercise training (Ng *et al.*, 2003). Following 13-14 weeks of training, aged rats had an upregulation of α_1 in RG but not EDL, increase of α_2 in RG, WG and EDL and increase of β_1 in EDL and RG, as measured by western blotting (Ng *et al.*, 2003). Training had no effect on β_2 abundance in any muscle group, whilst β_3 decreased after training in both red and white gastrocnemius muscles (Ng *et al.*, 2003). No studies have investigated the abundance of NKA isoforms and their adaptability with training in single fibres in older adults and this is investigated in Chapter 6.

2.5.6 Muscle [^{3}H]ouabain changes following exercise training in the elderly

Older adults who had been regularly been training between 12-17 years, completing swim, running or resistance training had a greater NKA, as measured by [^{3}H]ouabain binding, than untrained older adults (Klitgaard & Clausen, 1989). Interestingly, participants in the strength trained group also had greater NKA content than the young participants (Klitgaard & Clausen, 1989). The physical activity levels of the young participants were not stated, and this may have influenced this result. Further, the effect of a short-term exercise program on NKA content are unknown and is investigated in Chapter 6.

2.5.7 High-intensity exercise and training in the elderly

Both resistance and aerobic exercise training have important benefits for older adults. Resistance training is commonly used to increase muscle mass and strength and reduce the severity of sarcopenia, while aerobic training is used for the maintenance of metabolic and cardiovascular health (Nelson *et al.*, 2007). Twelve weeks of moderate intensity exercise training (60-80% heart rate reserve) in older adults also increased the CSA of type I fibres and improved muscle power and force production (Harber *et al.*, 2009; Harber *et al.*, 2012). There are advantages to high-intensity training (HIT) in place of moderate intensity long

duration training in healthy young populations (section 2.2.7), with moderate intensity aerobic training often used in older adults, presumably for participant safety. Regardless moderate intensity exercise training protocols improve aerobic power in active older adults typically by between 4.6- 9.0 $mL.kg^{-1}.min^{-1}$ (Suominen *et al.*, 1977; Seals *et al.*, 1984; Meredith *et al.*, 1989; Kohrt *et al.*, 1991; Coggan *et al.*, 1992; Konopka *et al.*, 2010).

Given the success of HIT in young adults, its use has recently been extended to diseased populations, with improvements in aerobic power ranging from 4-6 $mL.kg^{-1}.min^{-1}$ (Rognmo *et al.*, 2004; Broman *et al.*, 2006; Nemoto *et al.*, 2007; Wisloff *et al.*, 2007; Tomczak *et al.*, 2011). These are similar improvements to those seen after moderate intensity, training, but with reduced time commitments. Studies with elderly cardiac patients which used a protocol consisting of 4x4 minute exercise bouts at 90-95% peak HR achieved large improvements in VO_2 peak (46%), and muscle adaptations including upregulation in PGC-1α (47%) and rate of Ca^{2+} uptake by the SR (60%) (Wisloff *et al.*, 2007). These were greater than moderate intensity training which saw 14% improvement in VO_2 peak and no difference in muscle PGC1-α or rate of SR Ca^{2+} uptake (Wisloff *et al.*, 2007). A recent meta-analysis reported, four studies who had used HIT in patients with cardio-metabolic disease reported zero cases of adverse events resulting from high-intensity training; a further six studies which used HIT did not report whether adverse effects from training had occurred (Weston *et al.*, 2013). Surprisingly, no studies to date have investigated the effectiveness of HIT in healthy older adults. Given the evidence that HIT is safe and effective in improving aerobic power and also inducing some changes in skeletal muscle proteins, this training modality was investigated in older adults (Chapter 5).

2.6 AIMS

The aims of this thesis are to investigate the fibre-type specificity of the six NKA isoforms in human skeletal muscle fibres, using the single fibre western blotting technique, the adaptability of NKA isoforms within single fibres following different interventions, exercise training, physical inactivity and high-intensity interval training in the elderly, as well as the effects of ageing.

2.6.1 Study One

The aim of Study 1 was to explore whether the upregulation of NKA isoforms occurred in a fibre-type specific manner following RSE training.

2.6.2 Study Two

Study 2 aimed to investigate the effects of ageing on NKA isoform abundance in single fibres and on NKA content as measured by [^{3}H]ouabain binding, in healthy young and old adults.

2.6.3 Study Three

Study 3 aimed to investigate the effects of 12 weeks of high-intensity interval training (HIT) in aged individuals on NKA isoform abundance measured in single fibres and in whole muscle and further on [^{3}H]ouabain binding site content in healthy older adults.

2.6.4 Study Four

The aims of the final study in this thesis was to investigate the effects of 23 days of ULLS on the abundance of NKA isoforms in single fibres and to then explore the changes following a four week resistance period.

2.7 HYPOTHESES

2.7.1 Study One

Based on previous sprint training studies that demonstrated upregulation of NKA content in whole muscle and of β_1 isoform abundance in fractionated tissue, it was hypothesised that RSE training would increase the abundance of the α_1, α_2 and the β_1 isoforms in both fibre types and that these effects would be more pronounced in type II fibres due to the intense nature of the exercise.

2.7.2 Study Two

It was hypothesised that older adults who were matched for physical activity against young adults, would have a decreased α_2 isoform abundance and in [^{3}H]ouabain binding site content compared to healthy young adults. Based on findings from rodent studies it was hypothesised that the α_1, β_1, β_2 and β_3 would each be upregulated in older adults in a fibre-type specific manner that would not be detectable in a mixed muscle homogenate.

2.7.3 Study Three

It was hypothesised that 12 weeks of HIT in older adults would increase each of the relative abundance of the NKA α_1, α_2 and β_1 isoforms in single muscle fibres, the [^{3}H]ouabain binding site content and increase VO_2 peak. It was hypothesised that no changes would occur to the α_3, β_2 and β_3 isoform abundances.

2.7.4 Study Four

Given that the α_1, α_2 and β_1 isoforms are the major NKA isoforms typically upregulated by exercise training and markedly reduced by chronic spinal cord injury, it was hypothesised that 23 days of ULLS would decrease the abundance of the α_1, α_2 and β_1 isoforms in human skeletal muscle. As the α_3 and β_2 isoforms were not decreased by spinal cord injury it was hypothesised that these isoforms would be unaltered by ULLS Further, it was hypothesised

that resistance training would reverse any reduction of NKA isoforms caused by ULLS regardless of fibre-type.

CHAPTER 3. Single fibre co-expression and fibre-specific adaptability to short-term intense exercise training of Na^+, K^+-ATPase α and β isoforms in human skeletal muscle

3.1 INTRODUCTION

Repeated skeletal muscle contractions depend on the integrated regulation of neural, ionic and metabolic processes to ensure preservation of membrane excitability and force development. A key protein that underpins skeletal muscle excitability in sarcolemmal and t-tubular membranes, via Na^+/K^+ exchange and associated electrogenicity, is the Na^+,K^+-ATPase (Na^+,K^+-pump, NKA). The functional NKA unit comprises a heterodimer with a catalytic α and a regulatory β subunit, which are each expressed as multiple isoforms (NKA $\alpha_{1\text{-}4}$ and NKA $\beta_{1\text{-}3}$) and with each isoform likely to play differing roles in ion regulation (Blanco & Mercer, 1998).

Findings on the fibre-type specific expression of NKA α isoforms in various muscles in rats are inconsistent. Studies utilising isoform-specific antibodies have found both α_1 and α_2 isoforms to be similarly abundant in fast-twitch and slow-twitch muscle types (Hundal *et al.*, 1993; Fowles *et al.*, 2004; Kristensen & Juel, 2010). Opposing this, measurements conducted in muscles utilising [^{3}H] ouabain binding, which in rats selectively detects only the α_2 isoform, show a distinctly greater abundance in the EDL (fast-twitch) compared to soleus (predominantly slow-twitch) muscle (Clausen *et al.*, 1982). To date the fibre-type specific expression of α_3 has not been investigated. Reports in rodents on the NKA β isoform expression, using specific antibodies, consistently show fibre-specific differences, with a greater abundance of β_1 in oxidative and β_2 in glycolytic muscle (Thompson & McDonough, 1996b; Fowles *et al.*, 2004; Zhang *et al.*, 2006). A similar abundance of β_3 was reported in oxidative and glycolytic fibres of rat (Ng *et al.*, 2003).

In human skeletal muscle all NKA isoforms have been detected at the mRNA transcription level (Murphy *et al.*, 2004; Nordsborg *et al.*, 2005b) and all but α_4 at the protein level (Crambert & Geering, 2003; Murphy *et al.*, 2004). Human skeletal muscle is heterogeneous with respect to fibre type composition, comprising both slow-twitch, oxidative fibres (type I) and fast-twitch, oxidative or glycolytic (type II) fibres. Single fibre western blotting allows determination of fibre-specific protein expression in human skeletal muscle (Murphy, 2011). Recent work in human muscle has investigated fibre-type specific distributions of several NKA isoforms and demonstrated that α_1 and β_1 had a similar abundance in type I and II fibres (Thomassen *et al*., 2013). They also found that α_2 was more abundant in type II compared to type I fibres, but α_3, β_2 or β_3 were not investigated (Thomassen *et al.*, 2013). Given the diverse abundance of the NKA isoforms in rat and human skeletal muscle, any fibre-type specificity of various isoforms may have important functional implications, including modulation of NKA activity. Therefore the first aim of this study was to investigate fibre-type specificity of each of the six NKA isoforms in human skeletal muscle, using the single fibre western blotting approach.

Physical training is well known to increase NKA content in human skeletal muscle, as measured by [^{3}H]ouabain binding, which in human muscle binds with equal affinity to all three α isoforms (Clausen, 2013b). High-intensity "sprint" exercise training comprising multiple, maximal work bouts of 30 s or greater increases skeletal muscle NKA total content (McKenna *et al.*, 1993; Harmer *et al.*, 2006; Edge *et al.*, 2013). This training is typically associated with enhanced muscle and plasma K^+ regulation during exercise as well as improved exercise performance, suggesting important functional roles of NKA α upregulation (McKenna *et al.*, 1997; Harmer *et al.*, 2006; Iaia *et al.*, 2008; Thomassen *et al.*, 2010; Edge *et al.*, 2013). The effects of training comprising brief (≤ 6s) intermittent sprints with repeated efforts on the NKA isoforms in muscle are not well understood. A recent

repeated sprint exercise (RSE) training study in humans comprising 15 x 6 s sprint bouts for 8 weeks reported no change in the NKA α_1 or α_2 isoform abundance, but an increased β_1 in skeletal muscle (Mohr *et al.*, 2007). Interpretation of this data is difficult however, because the isoforms were measured in fractionated tissue, where the yield of NKA was unknown and thus not all NKA were measured (Hansen & Clausen, 1988). Three weeks of sprint or endurance training did not upregulate the α_1, α_2, β_1 isoforms in either SOL or EDL muscle of rat (Rasmussen *et al.*, 2011). However, 3 days following an acute bout of sprint exercise the NKA α_1, α_2, β_1 isoform abundances were increased in SOL, whereas no changes were observed in EDL (Rasmussen *et al.*, 2011). Whether fibre-type specific adaptations occur with intense intermittent training in human skeletal muscle is unknown. The second aim of this study was to determine fibre specific changes by investigating whether the abundance of the six NKA isoforms expressed in muscle is altered following four weeks of intense repeated-sprint exercise (RSE) training. Based on the previous sprint training studies that demonstrated upregulated NKA content in whole muscle and of β_1 isoform in fractionated tissue (Mohr *et al.*, 2007), it was hypothesised that the RSE training would increase the abundance of the α and the β_1 isoforms in both type I and type II fibres. Additionally it was hypothesised that these effects would be more pronounced in type II fibres due to the intense nature of the exercise and a greater recruitment of type II fibres than would normally be recruited in every-day activities undertaken by the participants.

3.2 METHODS

3.2.1 Participants and overview

Seventeen healthy young adults (10 male, 7 female) gave written informed consent and participated in this study, which was approved by the Victoria University Human Research Ethics Committee and conformed to the Declaration of Helsinki. Nine of these participants (4 male, 5 female) underwent a resting biopsy only and were only used in the fibre-type comparison (group 1). The physical characteristics of these subjects were (mean±SD) age 25.8±3.3 years, height 171.9±12.1 cm, mass 72.2±15.1 kg, peak oxygen consumption (VO_2 peak) 41.7±10.6 $mL \cdot kg^{-1} \cdot min^{-1}$. Eight of these individuals (6 male, 2 female) participated in a separate training study (group 2) and their physical characteristics were age 22.3±4.1 years; height 174.4±9.0 cm; mass 70.2±11.6 kg and 53.7±6.9 $mL \cdot kg^{-1} \cdot min^{-1}$ as described elsewhere (Serpiello *et al.*, 2011; Serpiello *et al.*, 2012). These subjects underwent a resting muscle biopsy prior to training (Pre) as well as forty-eight hours following the last RSE training session (Post, see section 3.3.2). Both males and females were included in the study as gender has been shown to have no effect on NKA [^{3}H]ouabain binding site content (Murphy *et al.*, 2007).

3.3.2 Repeat-Sprint Training

Participants trained three days per week for four weeks, on a non-motorised treadmill. Each training session comprised three sets of five, 4-s maximal sprints with 20 s of passive recovery with the sets of five spaced apart with four and a half minutes of passive recovery between the three sets, as previously described (Serpiello *et al.*, 2012). During the training period, participants were involved in their normal levels of recreational physical activity.

3.3.3 Muscle biopsy sampling and fibre separation

After an injection of a local anaesthetic into the skin and fascia (Xylocaine 1 %, AstraZeneca, Australia) a small incision was made in the *vastus lateralis* muscle and a sample was taken using a biopsy needle with suction.

For group 1 participants, who only underwent a muscle biopsy under resting conditions (n=9), the muscle was rapidly blotted on filter paper to remove excess blood and approximately 15 mg of muscle was taken and placed in a petri dish with paraffin oil. Approximately 30-40 single fibre segments (~3-5 mm in length) were separated from the fresh muscle under a dissecting microscope using jeweller's forceps.

Muscle samples collected from group 2 participants (n=8) were immediately frozen in liquid nitrogen and stored at -80 °C. Approximately 5-10 mg of this muscle was freeze-dried for 48 hours and brought to room temperature in a desiccator for approximately 30 minutes. The freeze-dried sample was placed in a Petri dish under a dissecting microscope and single fibre segments (~1-2 mm in length) were separated from the biopsy using fine jeweller's forceps (Murphy, 2011). Between 15-30 fibre segments were collected from each freeze dried biopsy sample.

All individual fibre segments from both fresh and freeze dried muscle were placed in separate microfuge tubes, containing 10 µl and 5 µl of 1x solubilising buffer respectively (0.125 M Tris-HCI, 10% glycerol, 4% SDS, 4 M urea, 10% mercaptoethanol and 0.001% bromophenol blue, pH 6.8) diluted 2:1 with 1 x Tris·Cl (pH 6.8) and stored at -80°C until western blotting analyses.

3.2.4 Single muscle fibre western blotting

Western blots were performed to determine the NKA isoform abundance and myosin heavy chain (MHC) protein expression in single skeletal muscle fibre segments. Given the longer length of the single fibre segments obtained from freshly collected muscle biopsies, it was

possible to divide each segment into two portions to analyse on two separate gels (i.e. 5 µl from the 10 µl total was loaded onto each gel), thus enabling all six NKA isoforms to be analysed in a single fibre segment. The western blotting technique employed was similar to that previously described (Murphy, 2011). Briefly, fibres were loaded onto gels with calibration curves (3 to 30 µg total muscle wet weight) derived from addition of graduated amounts of whole human muscle homogenate (Murphy & Lamb, 2013). Gels included 16-20 samples, comprising either 8 resting samples from two subjects (group 1) or 10 pre-training and 10 post-training fibre segments from the same one or two individuals (group 2). Denatured protein samples were separated on a 26 well, 4-15% Criterion TGX Stain Free gel (Bio-Rad Laboratories) and run for 45 minutes at 200 V. Using a wet transfer protocol, protein was transferred to nitrocellulose membrane at 100 V for 30 minutes. Membranes were incubated in Pierce Miser solution (Pierce, Rockford, IL) and blocked in 5% skim milk powder in Tris-buffered saline-Tween (TBST). Membranes were cut into three portions (>120 kDa, 120-70kDa, <70 kDa) and each section was incubated with antibodies diluted in 1% bovine serum albumin in phosphate-buffered saline with 0.025% Tween. Details of antibodies used are in Table 3.1. Membranes were incubated overnight at 4°C and 2 h at room temperature, all with rocking. After washing and incubating with a secondary antibody and following TBST washes, the membrane was coated with chemiluminescent substrate (West Femto, ThermoScientific, IL, USA). Typically when single fibres were split across two gels the $\alpha_{1\text{-}2}$ and $\beta_{1\text{-}2}$ isoforms could be probed for on one gel and the α_3 and β_3 probed on the second gel. This allowed all isoforms to be quantified in a single segment, despite each of the α isoforms migrating at ~100kDa and the β isoforms at ~50kDa. Specifically, the α_1 and β_1 antibodies are raised in mouse and α_2 and β_2 isoforms raised in a rabbit host, meaning the α_1 and β_1 isoforms could be probed for first, followed by the α_2 and β_2 and no cross-reactivity would occur. The α_3 and β_3 (both raised in mouse) were probed for on the second gel. This

provided the advantage that membranes did not need to be stripped, which could potentially remove some of the proteins embedded in the membrane. Because of cross-reactivity, in those cases where only one fibre was run on a gel (i.e. group 2), a maximum of 4 isoforms could be measured in a given fibre.

Images were taken using Image Lab software (Bio-Rad Laboratories). The positions of molecular mass markers were captured under white light prior to chemiluminescent imaging without moving the membrane. Membranes were then washed in TBST and re-probed using a different antibody with a different animal host.

Table 3.1. Antibodies used for human skeletal muscle single fibre Na^+, K^+-ATPase and myosin heavy chain isoform analysis. All antibodies were diluted in 1% bovine serum albumin in phosphate-buffered saline with 0.025% Tween.

Isoform	Primary antibody and source	Host species	Concentration
α_1	a6F, Developmental Studies Hybridoma Bank (DSHB), University of Iowa	mouse, monoclonal	1:750
α_2	anti-HERED, kindly donated by Professor Thomas Pressley, University of Texas	rabbit, polyclonal	1:200
α_3	MA3-915, Affinity Bioreagents	mouse, monoclonal	1:500
β_1	MA3-930, Affinity Bioreagents	mouse, monoclonal	1:500
β_2	06-171, Millipore	rabbit, polyclonal	1:500
β_3	610993, Transduction Laboratories	mouse, monoclonal	1:500
MHC I	clone A4.840, DSHB	mouse, monoclonal IgM	1:200
MHC II	A4.74, DSHB	mouse, monoclonal, IgG	1:200

3.2.4.1 Western blot data analysis

The 10-fold calibration curve on each gel showed good liner correlations ($r^2 > 0.90$). In order to maximise the number of fibres that could be used for analyses, a density value of 2-fold greater than the highest point on the curve was used as a cut-off point. It was felt this was reasonable as there was no indication of saturation on the curves used.

If the analysis was more stringent and excluded fibres from outside the calibration curve, this would have resulted in 19 fibres excluded from the final analyses. Before fibres which were outside the standard curve (but within double the density of the calibration curve), were included in the overall analysis, statistics for each isoform including and excluding these fibres from outside the calibration curve. There was no difference to the results when these fibres were included and thus these fibres were included in the overall analysis. It is important to remember fibres which were included from outside the standard curve were likely to be have underestimated the protein abundance and therefore this was undertaken to include as many data points as possible to gain a true representative of NKA isoform abundance in single fibres across a large number of participants.

Fibres that were greater than double the density of the calibration curve were excluded as the saturation point was not known. Also, given the majority of fibres excluded were dissected from freeze-dried tissue, it is plausible that multiple fibres were mistakenly collected as a single fibre.

The total number of fibres analysed is reported in Table 3.2. Another difficulty that has been described previously when collecting single fibre segments from a freeze-dried sample is that sometimes the fibre collection into the microfuge tube was not successful (Murphy 2011). If no protein was detected on the Stain Free gel then that ‘fibre’ then it was deemed that fibre was not successfully collected. Fibres that stained for both MHC I and II were also excluded

from data analyses (n=14) as it could not be differentiated whether this result indicated a hybrid fibre or that two fibres had been collected and placed in the same microfuge tube. Additionally, if a fibre did not stain for either MHC I or II, it was identified as a pure type IIx fibre and given the very low occurrence (n=2), these fibres were also excluded from further data analyses.

Stain Free gels were imaged before transfer (Stainfree Imager, Bio-Rad Laboratories, USA) and the density of total protein in each lane determined and used for normalisation for each protein (Murphy, 2011). Previous work has confirmed the amount of MHC which remained in the gel post-transfer was found to be a valid indication of the total protein loaded, with a linearity of the amount of MHC remaining in a gel following transfer reported at $r^2 > 0.98$ (Murphy *et al.*, 2006b).

Fibres analysed for each of the NKA isoforms were expressed relative to the total protein in each lane. To enable comparison of fibres across different gels, the fibres obtained from group 1 and the “pre” training fibres from group 2, which were collated to characterise the NKA isoform abundance within type I and II fibres, were normalised to the mean of type II fibres on that gel, as previously described (Thomassen *et al.*, 2013). The fibres collected from group 2 were normalised to the average type II pre-training fibres. Given that fibres from both group 1 and 2 were normalised to the same point on their respective calibration curves, then it was possible to pool data from the same individual across different gels. However the calibration curve which is run on each gel must be from the same whole muscle homogenate in order to compare across gels. Fibres from the two groups (1 and 2) were analysed at different stages of this PhD candidacy, and different homogenates were used for the respective calibration curves, meaning the data could not be pooled.

All gels run to investigate the effects of RSE on NKA isoform abundance in single fibres used the same homogenate to form the calibration curve and therefore to enable comparisons across gels, all fibres were normalised to the 5 µl point of the calibration curve. Each calibration curve had minimum a linear correlation of r^2= 0.90, which acted as a three point internal control for every fibre run.

In this study, the calibration curve did not contain a molar abundance and therefore western blots could not provide an absolute quantification of isoform abundance and it was not possible to correlate the varying abundances of isoforms within each fibre.

3.2.5 Alpha 2 antibody validation

A different α_2 antibody was used to the only published study that measured NKA in human single muscle fibres (Thomassen *et al.*, 2013). To enable comparison of our α_2 findings with that study an additional gel was run. The gel was loaded as two identical halves and contained homogenates from rat extensor digitorum longus (EDL) muscle, containing fast-twitch fibres, soleus (SOL) muscle containing predominantly slow-twitch fibres, brain and heart as well as a mixed human skeletal muscle homogenate prepared by mixing muscle homogenates from eight individuals. Following transfer and blocking, the membrane was cut in half vertically and each half incubated in either the anti-HERED α_2 antibody (Table 1) or the alternate commercially available α_2 antibody (1:500, rabbit, polyclonal, 07-674, Millipore).

Statistical Analysis

All data is expressed as mean ± SD. All data was tested for normality using Shapiro-Wilks test. All data was log-transformed before data analyses. To detect any fibre-type specific differences of NKA expression and training effects, a univariate nested model analysis (linear mixed model) was used with fibre-type as a fixed factor and participant as a random factor. This test was used as it calculates significance from the average of each isoform in each fibre-

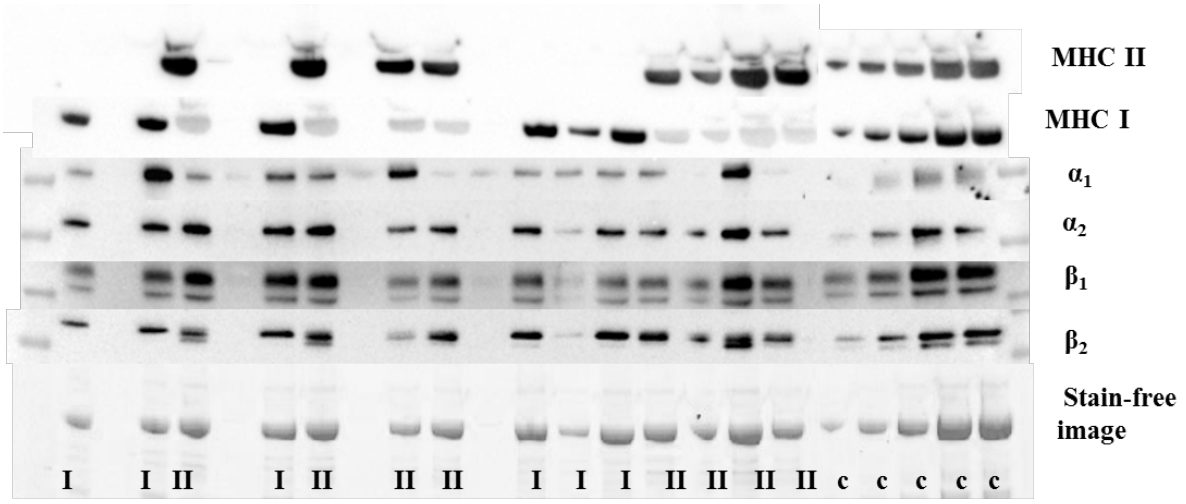
type from each participant, which is critical given the potential of large variation of protein abundance within one fibre-type from one person (Frankenberg *et al.*, 2013). The use of a traditional *t*-test analyses each fibre as an individual data point which would provide a false representation of the sample size used and does not take into account the biological variation within each fibre for that individual. The univariate provides a more accurate and stringent analysis than a *t*-test because it is able to take into account the individuals. As previous single fibre work (Thomassen *et al.*, 2013) used *t*-tests for the fibre-type comparisons and to enable comparisons of findings the characterisation of NKA isoforms specific differences in single fibres here also used both the univariate model and a student's independent *t*-test, allowing a more correct comparison. Statistical significance was accepted at $p < 0.05$. All statistics were run using IBM SPSS software version 20.

3.3 RESULTS

3.3.1 Co-expression and fibre-type specificity of NKA isoforms in single fibres

A key initial finding was that both type I and type II fibres expressed all six NKA isoforms known to be expressed in human skeletal whole muscle (Figure 3.1). Analyses of fibre-type specific expression via the univariate nested model indicated that the NKA α_1, α_2, β_1 and β_3 isoforms had no fibre-type specific expression (Table 3.2). The NKA β_2 isoform was 27% more abundant in type II fibres compared to type I ($p<0.05$), while there was a tendency for α_3 to be greater in type II fibres compared to type I ($p=0.058$). Analyses using *t*-tests showed similar results, with no difference between fibre types for α_1, α_2, β_1 and β_3. The only difference in the *t*-test was the finding that α_3 was 18% more abundant in type II fibres ($p<0.05$) and the β_2 was 33% more abundant in type II fibres compared to type I ($p<0.05$.).

A)



B)

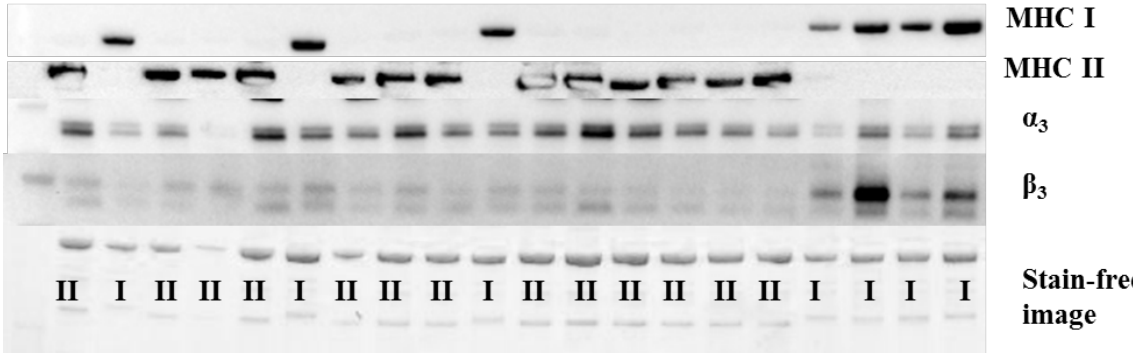


Figure 3.1 Representative blots of A) NKA α_1, α_2, β_1 and β_2, isoforms and B) NKA α_3 and β_3, isoforms in human skeletal muscle single fibre segments in human skeletal muscle single fibre segments. Band intensities in Stain Free image (bottom) indicate relative amount of tissue loaded in each lane and density was used to normalize respective Western blot band densities. Each lane is marked as either a type I (I) or type II (II) fibre. A five point calibration curve is shown on the upper gel (c). .

Table 3.2. Na^+, K^+-ATPase **(**NKA) isoform abundance determined in type I and type II single fibre segments in human skeletal muscle.

NKA Isoform	Type I	Type II
α_1	0.95 ± 0.45	0.97 ± 0.63
	(N=14, n=38)	(N=12, n=50)
α_2	1.03 ± 0.42	0.97 ± 0.35
	(N=16, n=61)	(N=15, n=63)
α_3	0.78 ± 0.49	0.94 ± 0.41 ‡†
	(N=9, n=34)	(N=10, n=39)
β_1	1.04 ± 0.58	1.06 ± 0.41
	(N=16, n=35)	(N=15, n=52)
β_2	0.77 ± 0.52	0.98 ± 0.44 *
	(N=11, n=27)	(N=12, n=45)
β_3	0.92 ± 0.61	1.11 ± 0.54
	(N=12, n=29)	(N=12, n=27)

Muscle was analysed from a total of 17 participants. For each isoform, n= total number of fibres analysed; N= number of participants whose biopsy data were included in that analysis. * $p<0.05$ greater in type II than type I by univariate nested model analysis and t-test, †$p<0.05$ greater in type II than type I by independent *t*-test ‡ $p\leq 0.06$ greater in type II than type I by univariate nested model. All fibres normalised to the average of type II fibres within each gel. Data is expressed as mean ± SD and in arbitrary units (a.u.).

3.3.2 Fibre-type specific adaptability to RSE training

For the analysis of training effects, within-subject comparisons of fibres only from the cohort of participants who completed training were used (N=8 group 2); the pre-versus post-training comparisons for NKA α and β isoforms are shown in Figure 3.2. For the NKA α isoforms, four weeks of RSE training did not modify the α_1 or α_2 abundances in type I or II fibres. A 60% non-significant increase of the α_3 was detected in type II fibres only ($p=0.101$). Analyses revealed that there was a between subject difference ($p<0.05$) suggesting that not all participants had an increased α_3 abundance.

For the NKA β isoforms, RSE training increased the β_1 abundance in type II fibres only ($p<0.05$), but the β_2 or β_3 isoforms were not affected by training.

3.3.3 Antibody testing

The comparison western blot showed that the α_2 band detected using the two different α_2 antibodies appeared at the same molecular weight (Figure 3.3). Additionally, both antibodies displayed similar relative amounts of α_2 between the tissues tested, with the highest α_2 expression in the brain homogenate, as expected for the α_2 isoform. Both antibodies detected a greater abundance of α_2 in the rat EDL compared with soleus muscle. This results suggest that the same protein band is analysed here as seen previously (Thomassen *et al.*, 2013).

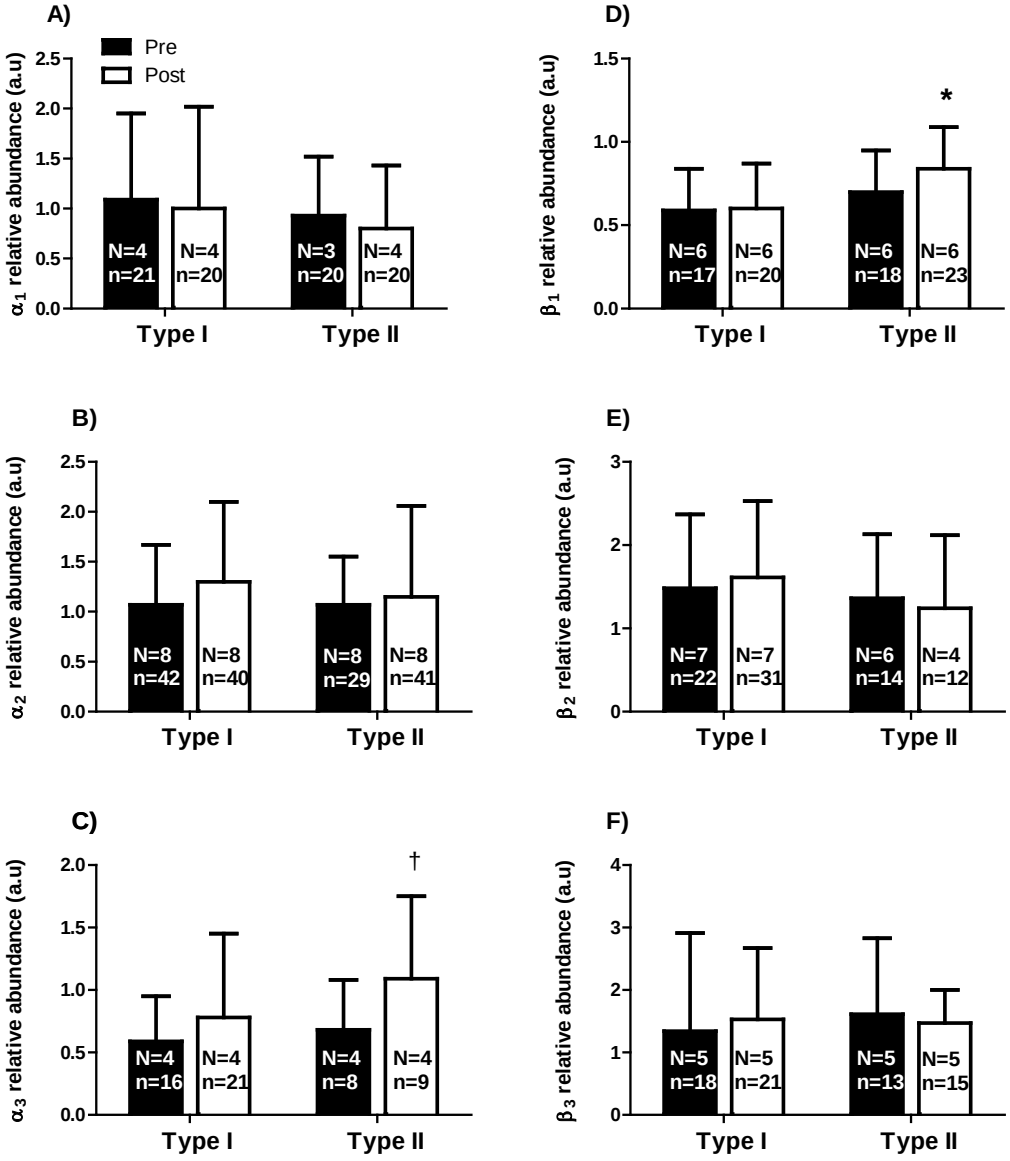


Figure 3.2 Na^+, K^+-ATPase A) α_1, B) α_2, C) α_3, D) β_1, E) β_2 and F) β_3 isoforms in human skeletal muscle type I and II single fibres before (Pre) and following four weeks of repeat sprint training (Post). Each isoform was normalised to the total protein in each lane and to the average of the pre-train type II fibres on each gel and expressed as relative abundance (a.u). Open bars denote Pre-training, filled bars denote Post-training. Number of subjects from whom biopsies were sourced (N) and the number of single fibres analysed (n) for each isoform are displayed in the bars. *$p<0.05$ Post greater than Pre.† $p<0.06$ tendency for Post greater than Pre. Data presented as mean ± SD.

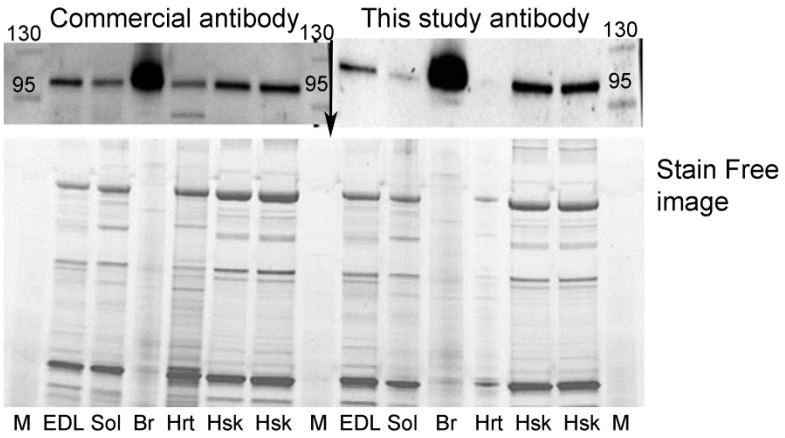


Figure 3.3 Representative blot of the α_2 isoform detected using two different antibodies. The left side of the gel contains the commercially available α_2 as used by Thomassen *et al.*, 2013, the right side contains the antibody described in Table 3.1. Both gels are loaded in the same order and each contains M) marker, EDL) rat extensor digitorum longus muscle, SOL) rat soleus muscle, Br) rat brain, Hrt) rat heart and Hsk) human skeletal muscle homogenate.

3.4 DISCUSSION

This study is the first comprehensive investigation of fibre-type specificity and training adaptations of all six NKA isoforms in human skeletal muscle and yields three novel and important findings. First, all six NKA isoforms (α_{1-3} and β_{1-3}) were expressed in both type I and II fibres. Second, and contrary to the hypothesis, which was based on findings from many studies in rat muscles, there was no fibre-type differences for five of the six NKA isoforms in muscle fibres obtained from this cohort of recreationally active, healthy young individuals. Only the β_2 isoform showed fibre-type specific expression with a higher abundance in type II fibres, which is also shown in rat skeletal muscle (Kristensen & Juel, 2010). Finally, intense RSE training induced fibre-type specific changes, with significant upregulation of the β_1 isoform in type II fibres, but with no changes in the α_1, α_2, α_3, β_2 or β_3 isoforms in either fibre-type. Whilst this study is able to show that each fibre expresses all six NKA isoforms, it is not possible to detect the ratio of each isoform in a given fibre because in order to make such a comparison then antibody binding efficacy must be equal and this is not known. Also, these data cannot determine whether these isoforms are co-expressed as functional units in the muscle. Importantly, all analyses were conducted on whole tissue samples using fibre segments without discarding any portion. This approach avoided the issues of repeated centrifugation of muscle to enrich membrane fractions, which typically result in low recovery of NKA and as such are likely to not be representative of the whole muscle NKA population (Hansen & Clausen, 1988).

3.4.1 Homogenous expression of the α isoforms in type I and II fibres in human skeletal muscle

There was no difference in NKA α_1 abundance between 38 type I and 50 type II fibres sampled from 14 individuals. This is consistent with a previous finding in human muscle (Thomassen *et al.*, 2013) and also with studies with rat muscle (Hundal *et al.*, 1993;

Kristensen & Juel, 2010). The α_1 isoform is reported to be ubiquitously expressed, binds Na^+, K^+ and endogenous ouabain, hydrolyses ATP and undergoes phosphorylation and oxidation (Clausen, 2003b; McKenna *et al.*, 2008) and in skeletal muscle is thought to play a "housekeeping" role in regulating trans-membrane Na^+/K^+ exchange under basal conditions (He *et al.*, 2001; Radzyukevich *et al.*, 2013). The high relative importance of α_1 in Na^+/K^+ exchange during the basal state, and its small role during muscle contraction were demonstrated in an α_2 skeletal muscle knockout mouse model, which showed an upregulation in the α_1 abundance with no impact on the resting membrane potential (Radzykevich *et al.*, 2013). The lack of fibre specificity in expression of α_1 in human skeletal muscle found here is consistent with this vital basal functional role, which would be required for all fibres. Surprisingly, there was no difference in the expression of the α_2 isoform between type I and II fibres. Our findings contrast with the only previous study using human skeletal muscle fibres, which reported a ~37% greater abundance of α_2 in type II than in type I fibres (Thomassen *et al.*, 2013). The reasons for the difference in α_2 isoform abundance between this and the recent study by Thomassen *et al.*, (2013) are unclear. The discordance between this and previous findings is unlikely due to the use of different antibodies as both seemingly detected the same bands in various tissues (Figure 3.4). Additionally, both antibodies detected the highest α_2 abundance in brain and also identified a greater relative abundance of α_2 in the rat fast-twitch EDL compared with predominantly slow-twitch soleus muscles. In rat muscle, the α_2 isoform has been reported to have similar abundance in muscles rich in fast or slow twitch fibres (Fowles *et al.*, 2004; Ingwersen *et al.*, 2011), although based on [^{3}H]ouabain binding site content measures, to be more abundant in muscles rich in type II fibres (Clausen, 2003b). In rat soleus skeletal muscle, the α_2 isoform comprises ~85% of all the α subunit isoforms (Hansen & Clausen, 1988; He *et al.*, 2001) and has a key role in Na^+/K^+ exchange during muscle contractions (Radzyukevich *et al.*, 2013). Therefore this study concludes that in the

recreationally-active, young, healthy subjects participating in this study, there was no difference in the relative abundance of α_2 in type I and II fibres.

Little is known about the NKA α_3 isoform abundance or its function in skeletal muscle. Here is it is demonstrated that α_3 abundance does not exhibit fibre-type specific expression in human skeletal muscle, but tended to be more abundant in type II fibres (univariate nested model, $p<0.06$; independent t-test, $p<0.05$). It has been suggested that during repeated contractions, the α_3 is activated when the α_1 and α_2 isoforms are working at capacity, acting as a standby isoform to assist in returning membrane potential after contraction (Blanco & Mercer, 1998). A non-major role of α_3 during muscle contraction is suggested following the finding that α_3 haploinsufficient mice had a similar time to fatigue and exercise capacity as control mice (Lingrel *et al.*, 2007). Further investigation into the functional roles of the NKA α_3 in skeletal muscle is warranted.

3.4.2 Fibre-specific expression of the beta isoforms in type I and II fibres

The functions of the β isoforms are less well known than those of the α isoforms. This is the first study to compare each of the β_1, β_2 and β_3 isoforms in human skeletal muscle single fibres and indicates a similar abundance in type I versus II fibres for the β_1 and β_3 isoforms. The β_1 data is consistent with a previous finding in human skeletal muscle (Thomassen *et al.*, 2013). Of interest, is that the lack of fibre-type specific expression for β_1 in human skeletal muscle data sharply contrasts with studies in rat muscle, which indicated distinct differences in β isoform expression between fibre types; these studies reported almost exclusive expression of the β_1 isoform in muscles rich in slow-twitch fibres (Hundal *et al.*, 1993; Thompson & McDonough, 1996a; Fowles *et al.*, 2004; Zhang *et al.*, 2006). This species difference in fibre specific expression for the β_1 isoform indicates that more research is required to provide an understanding into the functions of these isoforms. In human skeletal

muscle, the similar abundance of the β_1 isoform in type I and II fibres in human skeletal muscle suggests similar regulatory functional roles under basal conditions for all fibres. The β_2 was more abundant in type II fibres compared to type I, which is consistent with studies conducted in rats (Hundal *et al.*, 1993; Thompson & McDonough, 1996; Fowles *et al.*, 2004; Zhang *et al.*, 2006). Importantly, when group 2 was analysed separately to investigate the effects of RSE training, prior to training the abundance of β_2 appeared to be similar in type I and II fibres, which is in contrast to when group 1 and 2 data were pooled. This is likely due to a participant factor, as when group 1 were analysed separately the β_2 was significantly higher in type II fibres compared to type I. But the data when pooled together clearly shows the β_2 is more expressed in type II fibres. This suggests when determining fibre-type differences for NKA isoforms, a large sample of participants and fibres are required. This aspect of the analysis is discussed in more detail in the general discussion (Chapter 7). This is the first study in human skeletal muscle which has examined the fibre-type specificity of the β_2 and given the similar fibre-type expression is conserved across species it may be that the β_2 isoform plays a similar role in rat as in human skeletal muscle. The uptake of Na^+ and release of K^+ during both intermittent and continuous stimulation is greater in EDL compared to the SOL muscle, coinciding with earlier declines in muscle force and M-wave area (Clausen *et al.*, 2004). It may be possible that the β_2 has an important role in regulating NKA activity to negate the rapid Na^+/K^+ exchange in type II fibres. However detailed experiments investigating the function of the β_2 isoform are required to determine its function.

This is the first study to investigate the abundance of β_3 in human single skeletal muscle fibres, and the β_3 isoform findings of this chapter are similar to a single study conducted in rat skeletal muscle where no fibre-type difference was found (Ng *et al.*, 2003). Little is

known about the β_3. Similarly to the β_2 more investigation into the function of β_3 in skeletal muscle is required.

3.4.3 Effects of RSE training on NKA isoforms

RSE training affected only one of the six NKA isoforms expressed in human skeletal muscle single fibres, with β_1 isoform abundance significantly increased. Thus contrary to the hypothesis, the RSE training did not influence the abundance of either the α_1 or α_2 isoforms in type I or II fibres. A similar finding for NKA α_1 or α_2 isoforms measured in muscle biopsy extracts (i.e. mixed fibres) was found following a similar style of sprint training (Mohr *et al.*, 2007) and in moderately-trained runners after a high-intensity, reduced volume training (Gunnarsson & Bangsbo, 2012). Some studies have reported upregulation of α_1 and α_2 isoform abundance in human skeletal muscle after intensified training but in those studies the exercise bouts were of longer duration, lasting 30 s or more (Mohr *et al.*, 2007; Iaia *et al.*, 2008; Thomassen *et al.*, 2010). The muscle K^+ efflux is dependent on both intensity and duration of muscle contractions (Lindinger, 1995). Although the RSE training comprised "all-out" maximal efforts, the very brief contractions and intermittent nature of the exercise, probably enabled rapid recovery of any intramuscular K^+ disturbances, hence it is likely that any muscle K^+ loss/Na^+ gain were insufficient to elicit adequate stress to synthesise new α_1 or α_2 isoforms. Overall, it suggests that the training stimulus of this RSE training comprising very-short duration (4-s), high-intensity exercise bouts did not elicit sufficient physiological stress to upregulate these two main catalytic NKA isoforms. Previous research has shown that this RSE was sufficient to improve sprint exercise performance and also increased abundance of several skeletal muscle proteins associated with mitochondrial biogenesis (Serpiello *et al.*, 2011; Serpiello *et al.*, 2012).

RSE training induced an upregulation of the β_1 isoform in type II fibres. Similarly, upregulation of the β_1 isoform was also reported in mixed fibre muscle extracts following

short-duration, high-intensity training (Mohr *et al.*, 2007; Iaia *et al.*, 2011). It was earlier suggested that an increased β_1 abundance could support a higher NKA enzymatic activity based on measures of the α:β subunit ratio in purified plasma and intracellular membranes obtained from rat red skeletal muscle, in which membranes exhibiting a high β_1:α_2 ratio also had a higher NKA activity (Lavoie *et al.*, 1997). Therefore the upregulation of β_1 observed here may also have a beneficial impact on increasing NKA activity during intermittent exercise; however this remains to be determined. Both the β_2 or β_3 isoforms were not changed with RSE training. This finding is consistent with previous reports which demonstrates lack of adaptability after examined acute intense exercise (Murphy *et al.*, 2004), high-intensity interval training in well-trained cyclists (Aughey *et al.*, 2007), or spinal cord injury (Boon *et al.*, 2012). Together these findings suggest that the β_2 and β_3 isoforms are not as adaptable as other isoforms in response to chronic changes in physical activity. The functional significance of this is unclear but suggests more specialised roles for β_2 and β_3.

3.5 Conclusions

This study clearly demonstrates that NKA isoform expression in human skeletal muscle cannot be interpreted through the prism of findings obtained from studies in rat skeletal muscle, which demonstrate marked fibre-type specific expression, particularly for the β_1 isoform. In contrast, in a large number of single fibres it is shown clearly show no difference between the major fibre types for the two dominant α isoforms (α_1, α_2) and the major β isoform (β_1). The β_2 showed a greater abundance in type II fibres, as did the α_3 (when analysed using a *t*-test). The less well understood β_3 isoform also showed no significant fibre-type expression. Their different adaptability to chronic exercise further suggests different functional roles. Importantly, all six NKA isoforms were expressed in both type I and type II fibres.

Finally, with the single fibre western blotting technique, fibre-type specific adaptations were able to be determined following exercise training for the β_1 isoform. The detection of changes in NKA at the single fibre level is possible due to the reduction in inherent "noise" compared with assessing heterogeneous skeletal muscle. Future training studies should utilise single fibre western blots to investigate whether a higher volume, high-intensity training program induces upregulation of the other NKA isoforms and also how different variations of training can affect the upregulation of membrane transport proteins in different fibre types. The use of this technique has allowed detection of an NKA protein change with training in healthy young populations; and may also have important implications for detection of differences in muscle fibres from aged and diseased populations.

CHAPTER 4. The impacts of ageing on single fibre Na^+, K^+-ATPase isoform fibre type content and whole muscle [^{3}H]ouabain binding site content

4.1 INTRODUCTION

Skeletal muscle force production requires the propagation of action potentials (AP) into the t-tubular system of a muscle cell. Each AP comprises a small influx of Na^+ and efflux of K^+ (Sjøgaard, 1983) and the frequent AP propagation during repeated muscle contractions lead to substantial Na^+/K^+ fluxes, causing a depolarisation of the membrane (Sejersted & Sjøgaard, 2000). This depolarisation limits the rate at which AP are propagated into the muscle cell thereby limiting Ca^{2+} release from the sarcoplasmic reticulum (SR), and resulting in less force produced by skeletal muscle (Dutka & Lamb, 2007).

In skeletal muscle the NKA is a key protein in maintaining trans-membrane Na^+/K^+ gradients, thus playing a large role in the preserving cell excitability. Ageing is associated with a progressive loss of muscle function (Ballak *et al.*, 2014). Consequently, any reductions to key NKA isoforms and decreases in total NKA content with ageing may contribute to decreases in muscle function (Fleg & Lakatta, 1988; Ford *et al.*, 1993; Skelton *et al.*, 1994; McKenna *et al.*, 2012).

To date, only one study has investigated changes in NKA isoforms in skeletal muscle in ageing humans, and reported a decline in the NKA α_2 and β_3 isoforms relative abundance in whole muscle homogenate (McKenna *et al.*, 2012). In contrast studies in rats report age-related increases in NKA α_1 and β_1 isoforms and decreases in β_2 isoforms, in red and white gastrocnemius muscles (Sun *et al.*, 1999; Ng *et al.*, 2003). Given the fibre-type heterogeneity in human skeletal muscle (outlined in Chapter 3), adaptations in muscle that may occur in a fibre-dependent manner might not be detected using a whole muscle homogenate. To date, no

study has investigated NKA isoforms in single fibres isolated from skeletal muscle obtained from aged and young individuals.

Whole muscle NKA analyses utilising the [^{3}H]ouabain binding assay have found small but non-significant differences in NKA content between older and young adults, being slightly lower in the aged (Klitgaard & Clausen, 1989; McKenna *et al.*, 2012). In those studies, the physical activity levels between the young and old were not matched and this may have confounded the results, as the NKA is highly adaptable to physical training (Green *et al.*, 1993; McKenna *et al.*, 1993).

This study had two aims. The first was to investigate NKA isoform expression differences using western blotting in single skeletal muscle fibres and whole muscle homogenates from young and old adults, that were matched for weekly hours of physical activity. The second aim was to further investigate NKA content of skeletal muscle from young and old individuals using [^{3}H]ouabain binding. It was hypothesised that older adults matched for physical activity time completed per week would have a decreased NKA α_2 isoform abundance and this would coincide with a decrease in [^{3}H]ouabain binding site content compared to healthy young adults. Based from rodent studies, it was hypothesised that the α_1 and β_1 isoforms would be upregulated in older adults and the β_2 and β_3 isoforms would be lower in older adults and that these would occur in a fibre-type specific manner and be undetectable in a mixed muscle homogenate.

4.2 METHODS

4.2.1 Participants

This study was approved by the Victoria University Human Research Ethics Committee and conforms to the Declaration of Helsinki. An information for participants sheet was mailed out to potential participants who had enquired about the study (n= 90). After reading all information and obtaining medical clearance, 18 participants signed informed consent and commenced pre-screening tests. One participant could not complete the incremental exercise test due to health constraints and was required to withdraw from the study. A total of 17 healthy older adults (OLD, 10 male, 7 female) and 15 healthy young adults (YOUNG, 6 male, 9 female) finished the study. The physical characteristics of the OLD (mean ± SD) were age 69.4 ± 3.5 years (range 65-76 years), height 170.8 ± 10.4 cm and body mass 75.2 ± 13.0 kg and for the YOUNG were age 25.5 ± 2.8 years, height 173 ± 12.3 cm, body mass 72.9 ± 15.6 kg. Apart from age ($p<0.05$), these physical characteristics did not significantly differ between groups ($p>0.05$).

All participants completed an exercise risk factor assessment form and a physical activity questionnaire to determine current levels of self-reported physical activity. All older participants were also required to obtain a medical clearance prior to inclusion in the study. All young adults were recruited following the testing of the older adults so that the YOUNG cohort could be recruited with a similar number of self-reported hours of physical activity. There were no differences in the self-reported weekly physical activity levels between OLD and YOUNG (8.2 ± 4.4 vs. 6.8 ± 3.9 hr. respectively, $p=0.416$).

4.2.2 Experimental design

All participants visited the lab on three occasions. The first visit involved familiarisation with an incremental exercise test on a cycle ergometer. During the second visit, participants

completed a symptom-limited incremental exercise test to determine peak oxygen uptake (VO_2 peak) and during the third visit a resting muscle biopsy was taken.

4.2.3 Familiarisation

Participants performed a shortened version of a "signs and symptoms limited" incremental exercise test on an electronically braked cycle ergometer (Excalibur Sport, Lode, The Netherlands). All participants were fitted with a heart rate (HR) monitor and blood pressure was measured before and during exercise by auscultation using a standard mercury sphygmomanometer. Participants commenced cycling at 20 W at a cadence between 50-70 revolutions per minute (rpm), with workrate increasing every min by 20 W for males and YOUNG females and 10 W for OLD females. The familiarisation test was stopped when the participant indicated an RPE of 13 (OLD) or RPE of 17 (YOUNG) from a 20 point scale, corresponding to a reading of "somewhat hard" and "very hard" respectively (Borg, 1982). As participants ECG was not monitored, for the familiarisation test exercise was not continued beyond RPE 13 for OLD individuals.

4.2.4 Symptom limited incremental test (VO_2 peak)

Participants performed the incremental test on the same electronically braked cycle ergometer. Before, during and after the test cardiac rhythm and HR were continuously monitored via a 12-lead electrocardiogram. Blood pressure was taken manually at rest, throughout exercise and for up to ten minutes of recovery. The test started at 20 W for both males and females. Tests completed by older adults increased 20 W each min for males and by 10 W each minute for females until symptom-limited endpoint, defined as a rating of perceived exertion (RPE) of 17 (very hard) on the Borg scale (Borg, 1982). For YOUNG the test started at 20 W and increased 20 W each min until symptom-limited RPE (RPE=17).

During exercise participants breathed through a Hans-Rudolph 3-way non-rebreathing valve, with expired air passing through flexible tubing into a mixing chamber. Expired volume was measured using a ventilometer (KL Engineering Sunnyvale, California, USA) and mixed expired O_2 and CO_2 contents were analysed by rapidly responding gas analysers (S-3lA/II and CD-3A analysers, Ametek, PA, USA). The gas analysers were calibrated immediately prior to each test using commercially prepared gas mixtures and the ventilometer was calibrated prior to each test using a standard 3 L syringe.

4.2.5 Resting muscle biopsy and single fibre separation

The biopsy procedure was as described (Chapter 3). A total of 17 older adults and 13 young participants underwent a resting muscle biopsy taken from the vastus lateralis muscle. Single fibres were dissected from the biopsies obtained from 14 OLD and 13 YOUNG individuals.

4.2.6 Whole muscle homogenate

Whole muscle homogenates were prepared from OLD (n=8) and YOUNG (n=8) individuals. A small portion of whole muscle (15-30 mg), was accurately weighed and homogenized on ice (1:20 wt:vol) in Na-EGTA solution (165 mM Na^+, 50 mM EGTA, 90 mM HEPES, 1 mM free Mg^{2+} (10.3 mM total Mg^{2+}), 8 mM total ATP, 10 mM creatine phosphate, pH 7.10) with a protease inhibitor cocktail (PIC, Complete; Roche Diagnostics, Sydney, Australia). Immediately following this, the homogenate was diluted to 33 $\mu g^{-1}\mu l$ using x3 SDS solution (0.125 M Tris-HCI, 10% glycerol, 4% SDS, 4 M urea, 10% mercaptoethanol and 0.001% bromophenol blue, pH 6.8). Finally, samples were further diluted to 2.5 µg wet weight muscle.μl^{-1} samples using x3 SDS solution diluted 2:1 with 1 x Tris·Cl (pH 6.8), as previously described (Murphy *et al.*, 2011).

Portions from each OLD muscle homogenate were pooled together and the pooled sample used to create four point calibration curves for every gel. Use of the same homogenate for all

calibration curves for the samples in this chapter allowed comparisons of single fibres or whole muscle homogenates across gels. Individually, these homogenates were used to compare the NKA isoform abundances between OLD and YOUNG. Additionally, these homogenates were used to examine the relative abundances of the commonly used housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the total protein (using Stain-free gels (Bio-Rad Laboratories)) in OLD and YOUNG.

4.2.7 Western blotting

Western blots on single skeletal muscle fibre segments and whole muscle homogenate were performed to determine the relative protein abundances of NKA isoforms ($\alpha_{1\text{-}3}$, $\beta_{1\text{-}3}$) and GAPDH, and myosin heavy chain (MHC) I and II for fibre type identification. All antibodies were diluted in 1% bovine serum albumin in phosphate-buffered saline with 0.025% Tween and are shown in Table 4.1. The western blotting technique is described in Chapter 3 (3.2.4).

Table 4.1. Antibodies used for NKA, MHC and GAPDH analysis. All antibodies were diluted in 1% bovine serum albumin in phosphate-buffered saline with 0.025% Tween

Protein	Primary Antibody	Host Species	Concentration
NKA α_1	a6F, Developmental Studies Hybridoma Bank (DSHB), University of Iowa	mouse, monoclonal	1:750
NKA α_2	07-674, Millipore	rabbit, polyclonal	1:500
NKA α_3	MA3-915, Affinity Bioreagents	mouse, monoclonal	1:500
NKA β_1	MA3-930, Affinity Bioreagents	mouse, monoclonal	1:500
NKA β_2	06-171, Millipore	rabbit, polyclonal	1:500
NKA β_3	610993, Transduction Laboratories	mouse, monoclonal	1:500
MHC I	A4.840, DSHB	mouse, monoclonal IgM	1:200
MHC II	A4.74, DSHB	mouse, monoclonal, IgG	1:200
GAPDH	6C5 (ab8245) Abcam	mouse, monoclonal	1:10,000

Fibres were run on either 10% or 4-15% TGX Stain Fee gels. When loading gels the proportion of type I and type II fibres was not known and thus there are unequal numbers for different fibre-types.

In both OLD (n=8) and YOUNG (n=8), in a given single fibre segment was run across two gels (as described in Chapter 3) to allow all six NKA isoforms to be determined in the same single fibre. There were problems with the β_3 antibody for some gels, which could not be resolved and so a smaller sample size than for the other isoforms is presented.

4.2.7.1 Data normalisation

4.2.7.1.1 Single fibres

The same whole muscle homogenate was used as a calibration curve on all gels for this part of the thesis enabling comparisons of all these fibres across gels. Within a gel, each single fibre was normalised to the same point of the calibration curve (i.e. the 5 µl sample) of the 3-30 µg calibration curve. For all the NKA isoforms and the respective total protein (Stain Free) gels, curves showed a minimum linearity of $r^2 \geq 0.902$ and in most instances r^2 was $\geq$ 0.950. Some fibres were excluded from analysis if their density was twice the density of the largest point, as described earlier (Chapter 3).

4.2.7.1.2 Whole muscle homogenate

Whole muscle homogenates were analysed in three separate ways, to replicate methods used in several other studies in order to try to elucidate differences in reported results. Firstly, NKA isoforms in whole muscle homogenate were normalised to the commonly used housekeeping protein GAPDH. Secondly, isoforms were normalised to the total protein per lane and then to the 5 µl point of the calibration curve, and thirdly the data point for every sample was expressed relative to the same point on normalised against the raw data of the 5 µl point of the calibration curve (Murphy & Lamb, 2013).

4.2.8 [^{3}H]ouabain binding site content

Approximately 20 mg of muscle was used to measure the [^{3}H]ouabain binding site content, as previously described (Nørgaard *et al.*, 1984; Petersen *et al.*, 2012). Briefly, each sample was

washed for 2 × 10 min at 37 °C in vanadate buffer (250 mM sucrose, 10 mM Tris-HCl, 3 mM $MgSO_4$, 1 mM $NaVO_4$; pH 7.3). Following washing, samples were incubated for 2 h at 37 °C in vanadate buffer with the addition of [^{3}H]ouabain (2.0 μCi·ml^{-1} and 10-6M, PerkinElmer Inc., Boston, MA, USA). The muscle was then placed in ice cold vanadate solution for 4 x 30 min to remove any unbound [^{3}H]ouabain. Muscle samples were then blotted on filter paper and weighed before being soaked in 500 μL 5% trichloroacetic acid and 0.1 mM ouabain for approximately 20 hours. Following this, 2.5 mL of scintillation cocktail (Opti-Fluor, Packard, PerkinElmer Inc., Boston, MA, USA) was added before liquid scintillation counting of [^{3}H]ouabain. The content of [^{3}H]ouabain binding site content was calculated on the basis of the sample wet weight and specific activity of the incubation buffer and samples, and expressed as pmol.g wet wt^{-1}. The final [^{3}H]ouabain content was then calculated accounting for unspecific binding, correction factor for impurity of [^{3}H]ouabain, loss of bound [^{3}H]ouabain during washout and incomplete saturation, as previously described (Nørgaard *et al.*, 1984).

4.2.9 Statistical Analysis

All data are presented as mean ± SD. All data were tested for normality using the Shapiro-Wilks distribution test. All single fibre data was log-transformed. An independent student *t* test was used to determine differences between OLD and YOUNG for each of peak HR, workrate and VO_2 as well as for whole muscle homogenate western blot analyses for the NKA isoforms and for muscle [^{3}H]ouabain binding site content. A univariate nested model analysis (linear mixed model) was performed to detect any fibre type and age differences in single fibres. Fibre-type specificity of NKA isoforms in young and old were additionally measured by a student's independent *t* test, for comparative reasons noting the limitations in this approach described in Chapter 3. All statistics were performed on IBM SPSS statistics version 20.0. Significance was set at $p<0.05$.

4.3 RESULTS

4.3.1 Participant exclusion

For one older individual the exercise test was stopped prematurely due to a dangerously high systolic blood pressure of 270/90 mmHg being reached during exercise; these data were not included in analyses. As this occurred prior to the muscle biopsy procedure no muscle biopsy was collected from this participant.

4.3.2 Incremental exercise test performance

Lower values were found for OLD during incremental exercise than YOUNG for each of RPE limited VO_2 peak (23.6 ± 4.9 vs. 36.6 ± 6.5 $ml.kg^{-1}.min^{-1}$, 42%, respectively, $p<0.05$), HR peak (138 ± 13 vs. 172 ± 12 beats $minute^{-1}$, 21%, respectively, $p<0.05$) and peak workrate at RPE 17 (143 ± 46 vs. 226 ± 66 W, 44%, respectively, $p<0.05$).

4.3.3 Effects of ageing on NKA isoform abundance in single fibres

There were no differences between OLD and YOUNG for the α_1 or α_2 isoform abundance in either type I or II fibres (Table 4.2). There was 18% less α_3 NKA isoform in type II fibres in OLD compared to YOUNG ($p < 0.05$) but with no difference in type I.

The β_1 was not different between OLD and YOUNG in type I and II fibres. However, the β_2 isoform relative abundance was 52% lower in OLD compared to YOUNG in type I fibres ($p < 0.05$), and there was a tendency for β_2 to be also decreased in OLD in type II fibres ($p =0.051$). The β_3 was higher in type I fibres of the OLD compared to YOUNG ($p<0.05$, Table 4.2).

Overall, when single fibre data were analysed by a student's independent t-test the statistical analyses were similar to the Univariate model. There were, however, some differences with

the α_1 lower in OLD type I fibres compared to YOUNG ($p<0.05$). The β_1 was lower in OLD compared to YOUNG in type II fibres ($p<0.05$).

Table 4.2 NKA isoform abundance in vastus lateralis muscle in type I and II fibres obtained from young and old human skeletal muscle

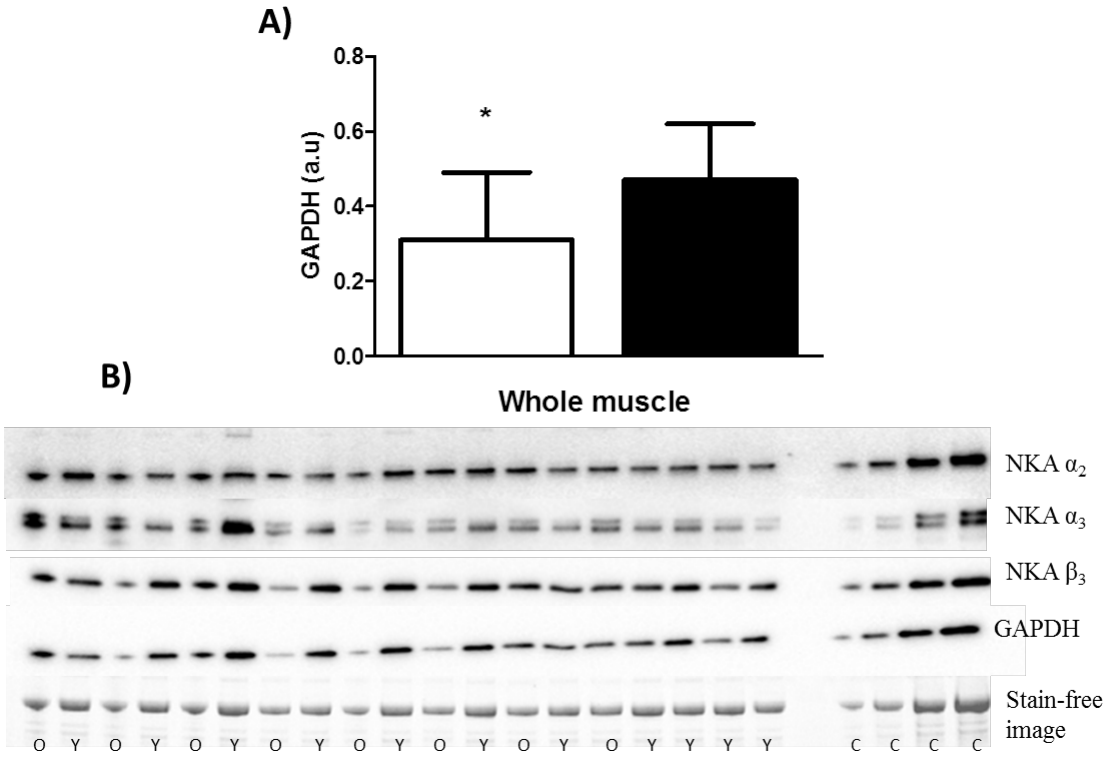
NKA Isoform	Type I		Type II	
	Young	Old	Young	Old
α_1	0.63±0.44 N=8, n=21	0.96±0.77 N=15, n=65	0.68±0.50 N=10, n=29	0.88±0.81 N=7, n=30
α_2	0.72±0.30 N=9, n=25	0.77±0.42 N=13, n=75	0.75±0.24 N=8, n=33	0.67±0.22 N=11, n=30
α_3	0.76±0.54 N=8, n=21	0.85±0.33 N=11, n=47	1.17±0.72 N=8, n=30	0.97±0.79 ****** N=6, n=19
β_1	0.87±0.50 N=9, n=24	0.75±0.24 N=13, n=59	0.96±0.58 N=8, n=35	0.70±0.23 N=11, n=27
β_2	1.15±0.47 N=8, n=17	0.67±0.50***** N=11, n=41	2.11±0.97 N=8, n=27	0.91±0.59† N=8, n=24
β_3	0.58±0.50 N=7, n=11	1.29±0.79* N=4, n=19	0.51±0.37 N=8, n=15	1.05±0.66‡ N=2, n=11

N= number of participants; n= number of fibres. * $p<0.05$ old different to young in the same fibre type † $p=0.051$ old < young in the same fibre type ‡$p<0.06$ old > young from same fibre type. All data were analysed using a univariate nested model analysis. All fibres are normalised against the total protein and then to the same point of the calibration curve. Data are expressed as mean ± SD and in arbitrary units (a.u.)

4.3.3 NKA isoform abundance in whole muscle homogenate in old and young

A representative blot of NKA α_2, α_3 and β_3 isoforms and GAPDH measured in whole muscle is presented in Figure 5.2.

Figure 4.1 A) GAPDH in whole muscle in OLD (n=8, open bars) and YOUNG (n=11 closed bars) B) Representative blots of whole muscle homogenate NKA α_2 α_3 and β_3 isoforms and GAPDH from YOUNG and OLD analysed by western blotting.



Analysed by independent *t*-test * old less than young ($p<0.05$). Data expressed as mean + SD. B) Representative blot of NKA α_2, α_3 and β_3 isoforms, O represents old, Y represents young, C represents calibration curve.

When measured in whole muscle homogenates and expressed relative to total protein there was a greater abundance of NKA α_2 in OLD compared to YOUNG ($p<0.05$, Table 4.3, first column), with no other differences between OLD and YOUNG. When normalised to GAPDH, however, the NKA α_2, α_3 and β_3 isoforms were greater in OLD, and there was a tendency for greater α_1 in OLD compared to YOUNG (Table 4.3). When taking into account the upper and lower limits of detection, as well as the total protein content, achieved by expressing NKA isoform density relative to the calibration curve (right columns in Table 4.3), the only difference between OLD and YOUNG seen was for NKA β_3, which was less in OLD compared to YOUNG (Table 4.3).

Table 4.3 Whole muscle homogenate western blot results for NKA isoforms α_{1-3} and β_{1-3} using three different normalisation methodologies.

NKA isoform	Age group	Normalised to Total Protein and calibration curve (a.u.)	P Value	Normalised to GAPDH (a.u.)	P Value	Normalised to Calibration Curve (a.u.)	P Value
α_1	YOUNG OLD	1.49 ±1.21 1.32 ±1.24	p=0.39	1.88 ±1.01 5.24 ±5.22	p=0.09	0.59 ±0.25 0.70 ±0.61	p=0.64
α_2	YOUNG OLD	0.67 ±0.16 0.83 ±0.16	p=0.05	1.71 ±0.77 3.89 ±2.09	p=0.01	0.45 ±0.11 0.41 ±0.99	p=0.43
α_3	YOUNG OLD	1.14 ±0.72 1.53 ±0.62	p=0.23	0.85 ±0.44 2.07 ±1.20	p=0.01	0.79 ±0.58 0.79 ±0.39	p=0.99
β_1	YOUNG OLD	0.81 ±0.16 0.82 ±0.20	p=0.64	5.80 ±1.89 10.49±6.59	p=0.11	0.47 ±0.09 0.45 ±0.16	p=0.73
β_2	YOUNG OLD	0.80 ±0.24 0.83 ±0.23	p=0.77	2.20 ±1.07 3.62 ±2.10	p=0.16	0.48 ±0.16 0.44 ±0.16	p=0.56
β_3	YOUNG OLD	1.04 ±0.29 0.78 ±0.34	p=0.12	2.20 ±0.26 2.95 ±0.57	p=0.01	0.70 ±0.22 0.41 ±0.18	p=0.01

All data is expressed in arbitrary units (a.u.) and presented as mean ± SD. NKA isoforms analysed from OLD (n=8) and YOUNG (n=11) participants and analysed by an independent *t*-test. Each isoform was separately normalised to the total protein of each lane (first column), the GAPDH abundance (second column) or the raw value at the 5 µl point on calibration curve which was run on every gel (third column). Representative blots can be seen in Figures 4.2.

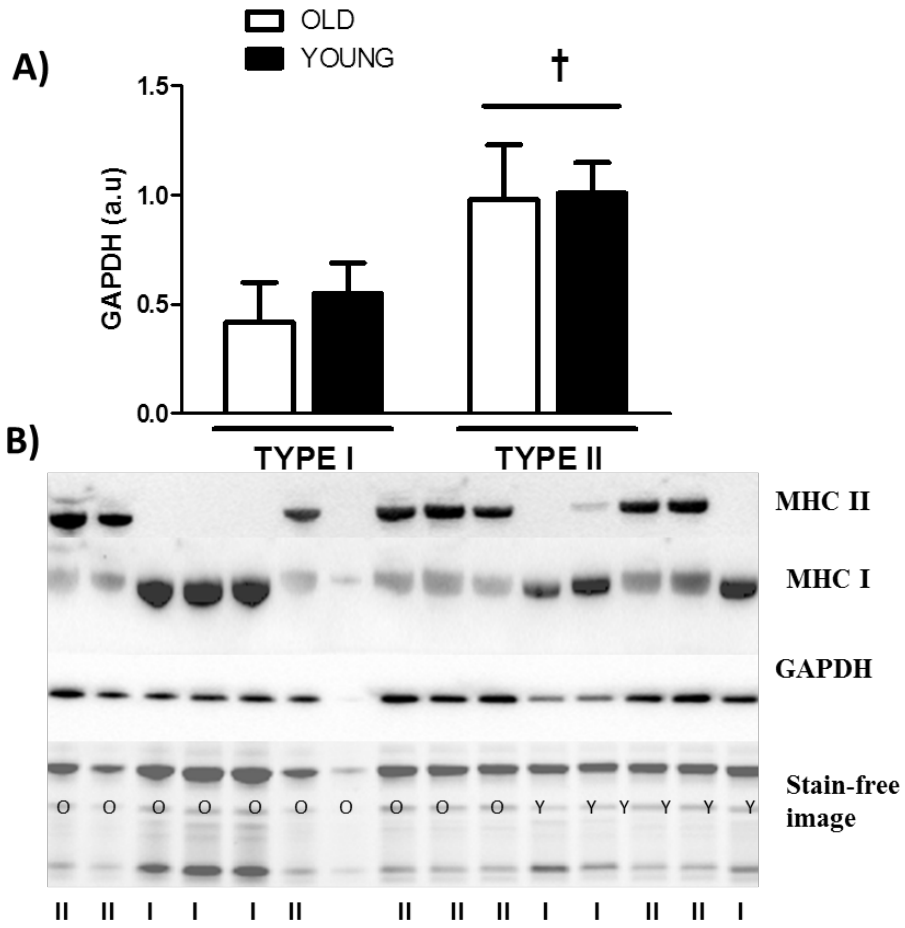


Figure 4.2 The relative abundance of GAPDH in A) type I and II fibres from OLD and YOUNG adults. Single fibres were analysed by an independent *t*-test for fibre-type and a separate test was run for age differences in GAPDH abundance † $p<0.05$, type II > type I. Data expressed as mean ± SD. B) representative blot of single fibres analysed for GAPDH, each lane shows one single fibre characterised as either a type I (I) or type II (II) fibre, fibre collected from O- OLD, Y -YOUNG.

4.3.4 Effects of ageing and GAPDH fibre-type specificity in human single fibres and whole muscle

When measured in single fibres, there was no difference in the abundance of GAPDH between YOUNG and OLD in either type I or II fibres (Figure 4.2).

However, fibre-type analysis revealed that both OLD and YOUNG had a greater GAPDH abundance in type II fibres compared to type I ($p<0.05$) (Figure 4.2).

In whole muscle homogenate, GAPDH was reduced in OLD compared to YOUNG, regardless of how the data were expressed (Figures 4.3 and 4.4). Further, comparison of the total protein (comprised predominantly of actin and myosin) was reduced 29% in OLD compared to YOUNG ($p<0.05$) (Figure 4.4) but when the sample size was increased to OLD (n=12) then this was not statistically significant ($p<0.2$).

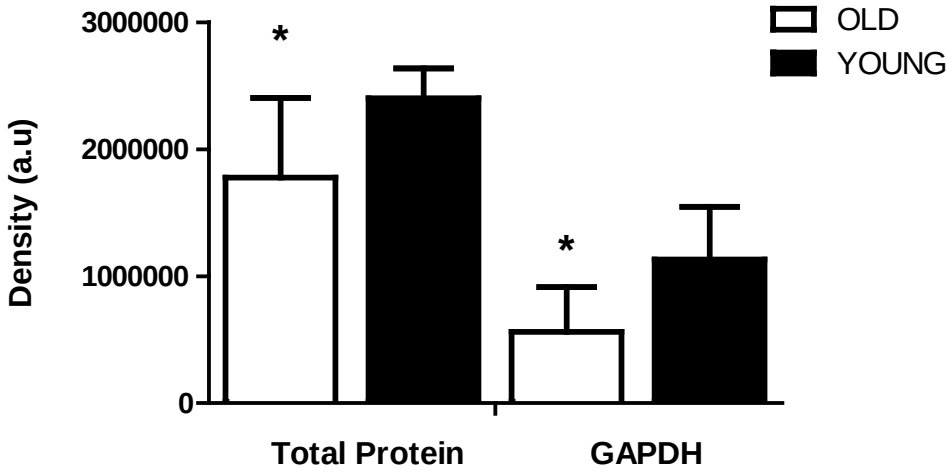


Figure 4.3 The raw data (density) for total protein and GAPDH measured in whole muscle homogenate from healthy young (n=13) and older adults (n=8).* $p<0.05$ old less than young. Data is expressed as mean ± SD.

4.3.5 [^{3}H]ouabain binding

There was no difference in muscle NKA content between YOUNG and OLD ($p=0.354$, Figure 4.5). When the OLD were separated into two different age groups based on the median age (65-69 (n=7) vs. 70-76 (n=10)) there was 15% less [^{3}H]ouabain binding in the oldest OLD group (394 ±57 vs. 339 ±50.13 pmol.g^{-}1, $p<0.05$). Intrestingly the older adults in

the 65-69 age group had 14% higher [^{3}H]ouabain binding than YOUNG (394 ±5 vs. 341.5 ±59.8 pmol.g^{-1}).

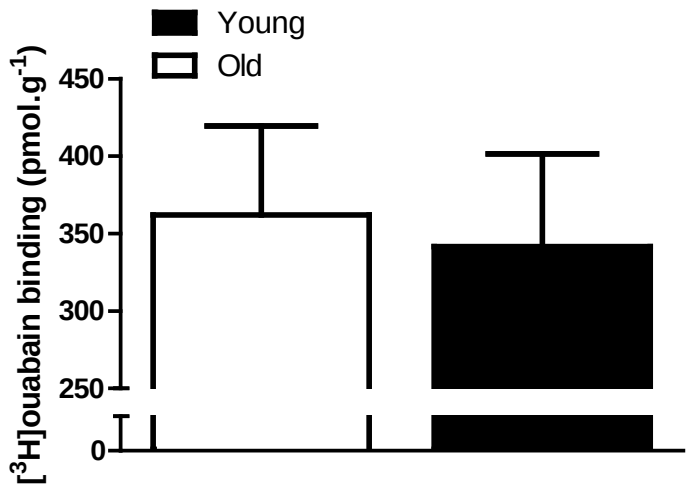


Figure 4.4 [^{3}H]ouabain binding site content in young (closed bars) and old (open bars). Data are presented as mean ± SD.

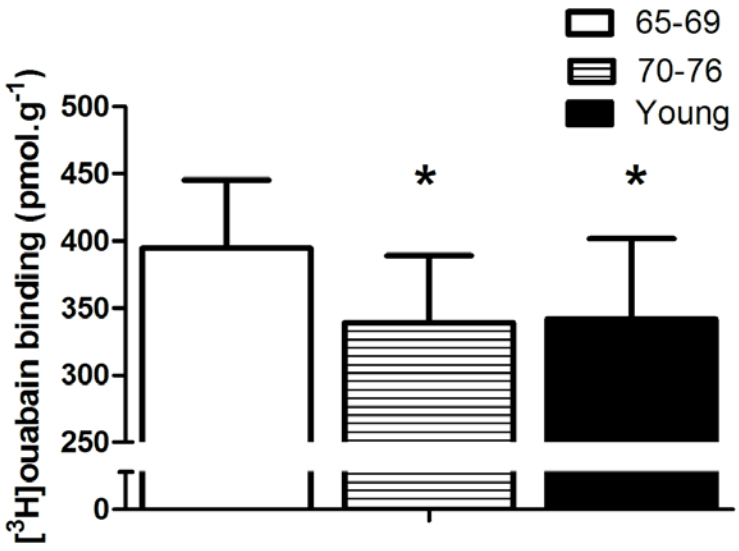


Figure 4.5 [^{3}H]ouabain binding site content in and 65-69 years (open bars), 70-76 years (pattern bars) and young (closed bars). * denotes less than 65-69 years. Data are presented as mean ± SD.

4.4 DISCUSSION

This is the first comprehensive investigation of fibre-type specific expression of NKA isoforms in skeletal muscle with ageing. This research yields two novel and important findings. Firstly, ageing was shown to differentially affect the abundance of skeletal muscle NKA isoforms in type I and type II fibres. Secondly, the use of GAPDH as the house keeping protein for normalisation produced differing results in NKA isoform abundance, indicating that GAPDH is inappropriate to use for this purpose.

4.4.1 Implications of changes of NKA isoform abundance in single fibres of older adults

An important finding in this study was that the NKA α_1 and α_2 isoform abundance in skeletal muscle were unaffected by ageing in both type I and II fibres. Given that these two isoforms are the most abundant α isoforms in skeletal muscle (Radzyukevich *et al.*, 2013) their expression has important implications in the maintenance of muscle function. Studies in rat red and white gastrocnemius and SOL muscle utilising immunohistochemical and immunoblotting techniques reported an upregulation of α_1 with or without a decline in α_2 abundance in ageing (Ng *et al.*, 2003; Zhang *et al.*, 2006). It is possible that participants in this study weren't sufficiently old to elicit declines in α isoforms, as seen in rat muscle. Rats aged between 24-30 months reportedly have the same skeletal muscle development as equal to or even greater than 80 year old humans (Grounds *et al.*, 2008), which is a common age used in rat studies (Sun *et al.*, 1999; Ng *et al.*, 2003; Reis *et al.*, 2005; Zhang *et al.*, 2006). In contrast, the mean age for participants in this study was 69 years ranging from 65-76 years. However, the α_3 abundance was lower in type II fibres from OLD compared to type II fibres in the YOUNG. The functional implication of this is unknown given the uncertainty about the function and the seemingly relative low abundance of the α_3 in skeletal muscle compared to other NKA isoforms (Clausen, 2003b).

The NKA β_1 abundance was also unaltered in ageing; this has previously been shown to be increased in white and red gastrocnemius muscle in 30 month old rats compared to 6 month old rats (Sun *et al.*, 1999; Zhang *et al.*, 2006), but with no differences seen between 16 and 29 month old rats (Ng *et al.*, 2003). Similarly to α_1 and α_2 isoforms, the age difference may not be sufficiently advanced enough to elicit age associated changes. Further, the β_1 has been shown to be adaptable to exercise training in both whole muscle (Benziane *et al.*, 2011) and in single fibres (Chapter 3); thus the similar duration of physical activity hours per week in the young and old groups has minimised any changes in the β_1 isoform with ageing.

There was a decrease in the relative abundance of β_2 present in type I fibres in both OLD and YOUNG. A decrease in β_2 has also been reported in red gastrocnemius (mixed) muscle, specifically type IIb fibres of old rats, as detected by immunohistochemistry (Zhang *et al.*, 2006) and in white gastrocnemius (predominantly type IIb fibres) via immunoblotting (Ng *et al.*, 2003). The functional implications of the decrease of the NKA β_2 isoform are unknown; findings from studies in rats suggest the exclusive abundance of β_1 and β_2 isoforms in different fibre-types, which may be related to the maintenance of membrane excitability in fast muscle (Zhang *et al.*, 2006). In Chapter 3 of this thesis, based on the higher β_2 isoform expression in type II fibres, it was speculated that the β_2 may have a specific function in regulation of NKA under large physiological stress, as there is a greater Na^+/K^+ exchange and reduction of membrane excitability in rat EDL compared to SOL muscle (Clausen *et al.*, 2004). A decrease of this isoform in type I fibres and tendency to decrease in type II ($p<0.051$), in skeletal muscle fibres in OLD may contribute to declines in the ability of Na^+/K^+ exchange and potentially contributing to an earlier impairment of E_m compared to young. However, before the true functional effects of these findings can be interpreted, the role of the β_2 isoform must firstly be determined in human skeletal muscle; which is beyond the scope of this thesis.

This study also found a greater β_3 isoform abundance in type I fibres in OLD compared to YOUNG. Studies in rats have also seen decreases in β_3 abundance in both red and white gastrocnemius measured via immunohistochemical and immunoblotting techniques (Ng *et al.,* 2003; Zhang *et al.,* 2006). The β_3 was not been measured in other muscles in rat studies and is only seldom measured in human studies, thus making interpretation of its function difficult. Training in aged rats decreased the abundance of β_3 compared to young rats, but no functional implications were discussed. Whether the β_3 decreases in human following training will be investigated in chapter 6 of this thesis.

4.4.2 No decreases in [^{3}H]ouabain binding in ageing

There was no change in [^{3}H]ouabain binding site content between OLD and YOUNG consistent with no change in either of the two key α isoforms (i.e. α_1 and α_2) with ageing. The decrease in the α_3 in type II fibres of OLD compared with YOUNG seemingly had no effect on NKA content, as determined by [^{3}H]ouabain binding in whole muscle, despite α_3 having the same affinity to cardiac glycosides as the α_1 and α_2 isoforms (Wang *et al*., 2001). A previous study in the aged separated the participants into cohorts more tightly aligned with respect to age and reported that participants aged 69-81 years had 26% less skeletal muscle [^{3}H]ouabain binding site content compared to those aged 55-68 years (Perry *et al.,* 2013). When the entire cohort was split in two differing age cohorts in the current study, there was a 15% reduction in [^{3}H]ouabain binding in the group 70-76 yr compared to the 65-69 year olds. A limitation of taking this approach is the reduced statistical power when the number of subjects being compared is decreased, although as they stand those results support findings from other studies (Perry *et al.*, 2013). It may also be possible that physical activity plays a large role in the preservation of NKA during ageing. In recreationally active young adults hours of physical activity completed per week was correlated to their muscle [^{3}H]ouabain binding content (Murphy *et al.*, 2007). Older adults who completed swim, running or

resistance training all had increased [^{3}H]ouabain compared to sendentary older adults; additionally older adults who regulary underwent resistance training had a greater NKA content than a young control group (Kiltgaard & Clausen, 1989). There was no difference between [^{3}H]ouabain binding between old and young who reported to complete a similar amount of physical activity per week (McKenna *et al.*, 2012). Thus it appears that physical activity may negate or delay the effects of ageing and maintain skeletal muscle NKA content. Interestingly, when 65-69 year old adults were compared against YOUNG there was a 14% higher [^{3}H]ouabain binding in older adults. This is likely to be caused by older adults expecting to have a larger muscle membrane area compared to YOUNG (McKenna *et al.,* 2012). It has been seen in porcine muscle that [^{3}H]ouabain binding was greater in smaller muscle fibres, which have a greater total membrane area of the muscle fibre (Harrison *et al.,* 1994). Fibre size was not measured in the current study, but it is well documented in the literature that type II fibres undergo atrophy in aged skeletal muscle (Lexell *et al.,* 1988) thus, the increased [^{3}H]ouabain binding seen in the elderly in this study may be related to fibre size and not NKA content per se. Additionally and in support of this finding, this study also found some evidence which may suggest that the total protein in aged muscle was decreased and thus the apparent no change of NKA α_1 and α_2 may in fact be overestimated and there may be more α_1 and α_2 isoforms in aged muscle compared to young. However more work is required before these claims can be made and is further discussed in 4.4.3.

4.4.3 Methodological considerations - Single fibre and whole muscle homogenate western blotting in ageing

The rationale for including whole muscle analysis in this study was to determine whether any fibre-type specificity of the NKA isoforms identified in a fibre specific manner with ageing would be detectable in a whole muscle homogenate. The results confirm that fibre-type specific changes to NKA isoforms are unlikely to be detected when whole muscle

homogenates are analysed. The use of calibration curves and loading small amounts of muscle (~12 μg) ensured there was no problem with sample saturation (Mollica *et al.*, 2009; Murphy & Lamb, 2013), which might have occurred in previous studies where 20-60 μg was loaded onto SDS-PAGE gels in previous NKA studies (McKenna *et al.,* 2012). However, no studies have specifically investigated saturation of the NKA isoforms in western blotting, and the assumption of saturation is based from studies in other proteins.

The single fibre results of this chapter were quite different to those using a whole muscle homogenate (McKenna *et al.,* 2012). That study found in whole muscle 24% lower α_2 and β_3 isoforms in an aged cohort. In contrast, in this chapter, single fibres showed no changes in either of these isoforms, with only a tendency for β_3 to be upregulated in type I fibres. This finding prompted the investigation into the house keeping protein GAPDH, which was used to normalise the whole muscle samples in previous work (McKenna *et al.,* 2012). This chapter found that GAPDH was more highly expressed in type II compared to type I fibres from both YOUNG and OLD individuals. Additionally, GAPDH protein abundance was lower in whole muscle obtained from OLD compared with YOUNG individuals. Previous work also in rats reported that GAPDH was decreased in type II fibres in 37 month old rats compared to 9 month old (Lowe *et al.,* 2000) and was 54% more abundant in type II fibres compared to type I (Galpin *et al.,* 2012). These findings demonstrate that GAPDH is an inappropriate protein to use for normalisation of proteins in heterogeneous skeletal muscle and in ageing, whether measured either in single fibres or in whole muscle homogenates.

The current study also revealed that based on wet weight of muscle, the total protein abundance in muscle from OLD was 29% lower compared with muscle from YOUNG. This was determined by accurately diluting the muscle in precise mass to volume ratios and so the wet weight of the tissue loaded was known. However, when a large number muscle

homogenates from older adults were analysed (n=12) there appeared to be no difference in total protein between young and old. The results presented for the NKA isoform abundance in single fibres in OLD may, in the most part, be likely underestimated because the NKA isoforms were typically lower in OLD compared with YOUNG. If the 29% lower total protein in OLD compared with YOUNG is considered, then it is possible that the present assessment of NKA isoform abundance in whole muscle is overestimated for the α_2 and underestimated for the β_3 isoform. However, given the differences in results between large and small sample sizes of older adults, the fundamental difference in the composition of muscle from OLD and YOUNG warrants further thorough investigation; however this was not undertaken during the course of this thesis.

When making fibre type comparisons for a given protein it is appropriate to express the protein of interest relative to the total protein present in the single fibre (as measured on the Stain Free gel), as the comparison is between the different fibres obtained from a muscle sample from the same individual. The use of the calibration curve necessarily takes into account methodological variations and limitations (Murphy& Lamb, 2013). Further implications and directions for future research for analysis and interpretation of western blotting in both single fibres and whole muscle of aged humans will be further discussed in Chapter 7.

4.5 CONCLUSIONS

This study demonstrates only selected fibre-type specific differences of the NKA to ageing where the α_3 isoform was lower in type II fibres and β_2 isoform lower in type I. Importantly, the key catalytic NKA α_1 and α_2 isoforms were unaffected by ageing when measured in single fibres. Furthermore, this was consistent with no difference found in total NKA content as measured by [^{3}H]ouabain binding site content. Finally, this study shows the use of

GAPDH to normalise NKA isoforms in whole muscle homogenate from ageing populations is not appropriate because it is both more highly expressed in type II compared to type I fibres and also because of the preferential reduction and atrophy of type II fibres in an ageing muscle compared to young (Lexell *et al.*, 1988).

CHAPTER 5 The effect of high-intensity interval training in older adults on Na^+, K^+-ATPase isoform abundance in single fibres and [^{3}H]ouabain binding site content

5.1 INTRODUCTION

Ageing is associated with decreased skeletal muscle size, aerobic power and muscle function, in addition to structural changes to skeletal muscle including decline of muscle fibre size and number of type II fibres (Lexell *et al.*, 1988). Despite these maladaptations, aged skeletal muscle can still adapt to exercise training at both a whole muscle and single fibre level (Konopka *et al.*, 2010). Both resistance (RT) and aerobic exercise training are important to older adults; RT to increase muscle mass, strength and to reduce the severity of sarcopenia; whilst aerobic training is beneficial for the maintenance of metabolic and cardiovascular health (Nelson *et al.*, 2007). High-intensity interval training (HIT) has been used in healthy young populations as it provides greater improvements than moderate intensity, continuous exercise training for improvements in aerobic power and in the abundance of muscle membrane proteins including NKA isoforms (Mohr *et al.*, 2007; Iaia *et al.*, 2008; Gibala *et al.*, 2012; Gunnarsson & Bangsbo, 2012).

In recent years, HIT has been applied to other populations including healthy middle-aged, older adults as well as patients with cardiovascular and metabolic diseases (Wisloff *et al.*, 2007; Tjønna *et al.*, 2013). The use of a short-duration HIT protocol comprising four minute efforts, has been used with no reported adverse events in older adults suffering cardiac complications, (Wisloff *et al.*, 2007; Weston *et al.*, 2013). Importantly, HIT in elderly cardiac patients increased skeletal muscle PGC-1α abundance by 47% in whole muscle and maximal Ca^{2+} uptake into the SR by Ca^{2+}-ATPase by 60% in skinned single muscle fibres, as well as improved aerobic power (Wisloff *et al.*, 2007). This indicates that both large cellular and whole body adaptations can occur from the use of HIT.

The effects of HIT on skeletal muscle NKA in whole muscle or in single fibres in older adults have not been measured. The NKA is important in regulating cellular Na^+/K^+ exchange, membrane potential and thus has an important role in muscle function. Given NKA content, as measured by [^{3}H]ouabain binding site content, was increased in whole muscle pieces following intensified training in healthy young people (McKenna *et al.*, 1993), it is important to determine whether NKA upregulation also occurs following HIT in the elderly.

One study showed older adults who had been training for 12-17 years had a higher NKA content than untrained older adults (Klitgaard & Clausen, 1989). The effects of a short-term exercise program on [^{3}H]ouabain binding and NKA isoform abundance in older adults are unknown. One training study in aged rats found that 13-14 weeks of training led to fibre-type specific upregulation of the NKA α_1, α_2 and β_1 isoforms and also with β_3 isoform decreased in both red and white gastrocnemius muscles (Ng *et al.*, 2003). Given the importance of NKA to muscle function and the fibre-type specific upregulation of NKA isoforms in aged rats, it is important to determine whether fibre-type specific upregulation of NKA isoforms also exists in an aged human population.

Therefore, this study had three main aims. Firstly, to determine the effects of 12 weeks of HIT on skeletal muscle NKA isoform abundance in single fibres in a cohort of older adults. Secondly, this study investigated whether [^{3}H]ouabain binding is increased with HIT and thirdly, to investigate the effects of HIT on exercise performance in the elderly. It was hypothesised that 12 weeks of HIT in older adults would increase the relative abundance of each of the NKA α_1, α_2 and β_1 isoforms in single muscle fibres, the [^{3}H]ouabain binding site content in muscle pieces and in VO_2 peak during incremental cycling exercise.

5.2 METHODS

5.2.1 Participants

Twenty older adults volunteered and gave signed informed consent for the study; seventeen passed the initial pre-screening assessments and fifteen of these participants completed the study. Physical characteristics of the 15 older adults who completed the study were; age 69.4 ± 3.5 years, height 170.8 ± 10.4 cm, body mass 75.2 ± 13.0 kg and BMI 21.69 ± 2.65 $kg \cdot m^{-2}$ (mean ± SD); their self-reported physical activity duration was 8.2±2.6 hours per week (as reported in Chapter 4). The study was approved by the Victoria University Human Research Ethics Committee and conforms to the Declaration of Helsinki.

5.2.2 Experimental design

Participants were randomised into either a training (n=10, HIT) or control (n=7, CON) group, but only 8 participants successfully completed the training. Before (Pre) and following the training or control period (Post) participants completed the following testing: an initial familiarisation session, a second visit to complete a "signs and symptoms limited" incremental exercise test to determine peak oxygen consumption ($VO_{2\ peak}$) and a third session when a resting muscle biopsy sample was taken.

5.2.3 Familiarisation

Participants performed a shortened version of a "signs and symptoms limited" graded exercise test on an electronically cycle ergometer (Excalibur Sport, Lode, The Netherlands). Participants were fitted with a heart rate (HR) monitor and blood measure was measured before and during exercise by auscultation using a standard mercury sphygmomanometer. Participants commenced cycling at 20 W at a cadence between 50-70 revolutions per minute (RPM), with workrate increased every min by 20 W for males and 10 W for females. As participants were not fitted with ECG for the familiarisation, to avoid a potential adverse risk

exercise was not continued beyond RPE 13, corresponding to a reading of “somewhat hard” from a 20 point scale(Borg, 1982).

5.2.4 Signs and symptom limited graded incremental test

The test was completed on an electronically braked cycle ergometer and HR peak, workrate peak and oxygen consumption peak (VO_2 peak) were measured, as detailed in Chapter 4. For participants’ safety, blood pressure was measured every 3 minutes during exercise by auscultation using a standard mercury sphygmomanometer.

5.2.5 Training and control groups

Participants trained under supervision three times per week for 12 weeks on a mechanically braked cycle ergometer (Monark 868, Vansbro, Sweden). A standardised 3 min warm up was conducted prior to every training session, after which participants were given one minute of passive rest on the cycle ergometer before the training session commenced. The training protocol was similar to a previously described protocol (Wisloff *et al.*, 2007) and comprised four, 4-minute intervals performed at an intensity corresponding to 90-95% of the HR peak attained during the incremental exercise test, with each interval interspersed by four minutes of active recovery where participants cycled at 50-60 % of peak HR. Each training session was followed by a 5 minutes cool down. Throughout the training session heart rate was recorded by a heart rate monitor (RS800sd, Polar Electro Oy, Kempele, Finland). Progressive overload was implemented into the training program, by increasing workrate in order for participants to reach the target HR as participants became accustomed to the exercise. Blood pressure was recorded at rest, during the final min each exercise bout and in the final min of recovery. Participants in the control group were asked to continue with their regular daily activities for 12 weeks, after which they also received 12 weeks of HIT; this was as an

incentive for participants to remain in the CON group for the entire period. No data or measures were taken from the CON group following the period they had completed HIT.

5.2.6 Resting muscle biopsy and single fibre separation

A resting muscle biopsy was taken pre and 48-72 hr following the final training session. The muscle biopsy procedure and single fibre separation from fresh tissue was identical to that described earlier (Chapter 3).

5.2.7 Whole muscle homogenate preparation

A whole muscle homogenate was prepared as described earlier (Chapter 4). This homogenate served two purposes. Firstly a small portion was used to generate a four point calibration curve for every gel. Second, western blots were performed on whole muscle homogenate to determine whether any training effects detected in muscle single fibres were also detectable in a whole muscle homogenate.

5.2.8 Western Blots

Western blots were performed to determine the NKA isoform and myosin heavy chain (MHC) protein abundance in single skeletal muscle fibre segments. In five out of the eight participants each fibre was halved and run across two separate gels for the analysis of all isoforms within the same cell, as described earlier (Chapter 3). Additional western blots were performed in whole muscle homogenate for NKA isoforms that were found to be upregulated in single fibres by training. To further demonstrate the effectiveness of the training, the mitochondrial protein cytochrome c oxidase (COX IV) was analysed in whole muscle samples. The western blotting technique was identical to that performed in Chapters 3 and 4. The antibodies used are shown in Table 4.1, with the addition of the COX IV antibody (#4850, Cell Signalling, Rabbit, Monoclonal, 1:1,000).

Each gel contained 10 Pre and 10 Post fibres; when loading fibres it was unknown whether these fibres expressed the MHC I or II isoform and thus an uneven number of type I and II fibres from before and after training were loaded. In some instances there were no type II fibres on a gel and the sample size for type II fibres was reduced compared to type I fibres.

5.2.8.1 Western blotting analysis

Each single fibre was normalised to the same point (5 µl) within the calibration curve, which spanned from 3-30 µg of protein. The linearity of each gel showed a minimum $r^2 \geq 0.9465$, except on two instances the r^2 was reported at 0.808 and 0.745, in these instances no more than 8 single fibres fitted the criteria for inclusion in the analysis for their respective isoform (as described in Chapter 3).

Where western blotting was conducted utilising whole muscle homogenates, samples used to detect NKA isoforms were normalised to the total protein per lane and then to the 5 µl point on the standard curve.

5.2.9 [^{3}H]ouabain binding site content

The muscle [^{3}H]ouabain binding assay was used to measure NKA content in whole muscle pieces, using the methods reported (Chapter 4).

5.2.10 Statistical Analysis

Single fibre data was not normally distributed and was log-transformed before analysis, while all other data was normally distributed and were run as raw data. A two-way repeated measures ANOVA was used (time×group) to compare peak HR, peak workrate, VO_2 peak and [^{3}H]ouabain binding between HIT and CON. A univariate nested model analysis (linear mixed model) was performed to detect any fibre type specific training differences in single

fibres. All statistics were performed on SPSS statistics version 20.0. All data are presented as mean ± SD. Significance was set as $p<0.05$.

5.3 RESULTS

5.3.1 Physical Characteristics of older adults training vs. control at baseline

At baseline there were no significant differences between HIT and CON in any of age, BMI, VO_2 peak or peak workrate (Table 5.1).

5.3.2 Compliance to training and adverse responses occurring from HIT

All participants completed a minimum 83% (30 out of 36) of training sessions. Due to an abnormally high blood pressure response to exercise in one participant and ill health in another, a total of two participants discontinued the training program and their data has not been included here. Testing and training were generally well tolerated by older adults. During the course of training five out of ten participants experienced vasovagal episodes. These incidents only occurred within the first two weeks of training. Only five participants in CON completed a Post biopsy.

5.3.3 The effects of HIT on body mass, VO_2 peak and exercise performance

There were no changes in body mass, blood pressure or BMI in either the training or control groups (Table 5.1). Following HIT, there was a 25% increase in peak workrate ($p<0.05$) and a 16% increase in VO_2 peak ($p<0.05$), with no changes in CON. There was also a tendency to a 5.6% increase in the HR peak after HIT ($p=0.07$, Table 5.1).

5.3.4 Isoform abundance in single fibres following HIT

The NKA α_1 abundance did not change following HIT in either type I or II fibres (Table 5.2). The α_2 was unchanged in type I fibres but was increased by 30% in type II fibres ($p<0.05$). The α_3 abundance was unchanged following HIT in type I and II fibres.

The NKA β_1 abundance was unaffected by training in type I or II fibres. The β_2 isoform relative abundance was increased after HIT by 52% in type I fibres ($p<0.05$), but was unchanged in type II. The β_3 was decreased after HIT by 48% in type I fibres ($p<0.05$) and by 20% in type II fibres ($p<0.05$) (Table 5.2).

5.3.5 Isoform abundance in whole muscle pre-post training

Mixed muscle homogenate analyses revealed no changes following 12 weeks of HIT in the NKA α_2 abundance (Pre-train 0.92±0.30 vs. Post-train 0.95±0.29 a.u) or β_2 isoform abundance (Pre-train 0.71±0.40 vs. Post-Train 0.87±0.23). Analyses of whole muscle homogenates showed a 21% increase abundance of the mitochondrial protein COX IV following training (0.93±0.56 vs. 1.17±0.55, $p<0.05$).

5.3.6 [^{3}H]ouabain binding

Following training there was a trend for NKA to be increased ($p<0.07$), with no change seen in CON (Figure 5.1). However, inspection of individual data revealed that five of the eight participants did respond to training with an increased NKA content. If statistics was run separately on these five people then the NKA content was increased following HIT (379.4 ± 43.16 vs. 438.0 ± 29.5, pmol.g wet weight^{-1}15%, $p<0.05$) (Figure 5.2).

Table 5.1 Physical characteristics and performance measures of healthy older adults before and after 12 weeks of high-intensity interval training or control.

Characteristic	Group	Pre	Post
Age (years)	HIT	69.8 ± 3.8	-
	CON	68.1 ± 3.3	-
Body Mass (kg)	HIT		75.78 ± 10.24
	CON	75.8 ± 10.24	74.9 ± 10.27
Stature (cm)	HIT	170.2 ± 8.4	-
	CON	169.4 ± 11.8	-
BMI ($kg \cdot m^{-2}$)	HIT	21.73 ± 2.2	21.82 ± 2.6
	CON	21.89 ± 3.2	22.14 ± 3.3
Resting systolic blood pressure (mmHg)	HIT	134.0 ± 17.0	125.6 ± 10.3
	CON	132.0 ± 12.0	136.0 ± 16.0
Exercise time (min)	HIT	7.3 ± 3.7	9.4 ± 4.5
	CON	9.63 ± 3.16	9.7 ± 3.1
Peak HR (bpm)	HIT	136 ± 16	144 ± 14‡
	CON	141 ± 11	142 ± 14
Peak power (W)	HIT	145.0 ± 49	181 ± 52†
	CON	142.0 ± 46.4	147.1 ± 40.2
VO_2 Peak ($ml.kg^{-1}.min^{-1}$)	HIT	24.7 ± 5.4	28.7 ± 5.1†
	CON	23.6 ± 5.3	23.8 ± 5.3

All data expressed as mean ± SD. N=8 for all data in trained group. Control group N=7 for all data except VO_2 peak where N=4. † Post > Pre $p<0.05$, ‡ Post tended > Pre, $p=0.077$.

Table 5.2 Na^+, K^+-ATPase isoform protein abundance in type I and II single fibres before (Pre) and following (post) 12 weeks of high-intensity training in older adults. Control data not analysed for single fibres.

	Type I		**Type II**	
Isoform	**Pre**	**Post**	**Pre**	**Post**
α_1	1.26±1.05 N=8, n=43	1.19±1.75 N=8, n=31	1.09±1.21 N=5, n=20	1.18 ±1.26 N=8, n=30
α_2	0.83 ±0.49 N=8, n=49	0.85 ±0.36 N=8, n=39	0.69±0.25 N=5, n=14	0.90±0.40 † N=8, n=38
α_3	1.00±0.51 N=5, n=22	0.86±0.36 N=6, n=17	0.74±0.40 N=3, n=8	0.86±0.40 N=5, n=21
β_1	0.84±0.30 N=7, n=38	0.96±0.35 N=5, n=37	0.81±0.22 N=5, n=14	0.93±0.46 N=7, n=29
β_2	0.75±0.46 N=5, n=20	1.14±0.65 † N=5, n=25	1.19±0.66 N=4, n=11	1.54±1.44 N=5, n=28
β_3	1.44±1.10 N=2, n=7	0.74±0.44 ‡ N=2, n=7	1.58±1.93 N=3, n=12	0.78±0.36 ‡ N=3, n=15

n, number of fibres; N, number of participants. † Post greater than Pre $p<0.05$ ‡ Post less than Pre $p<0.05$. All data analysed by an univariate nested model analysis. Data is expressed as mean ± SD and in arbitrary units (a.u.).

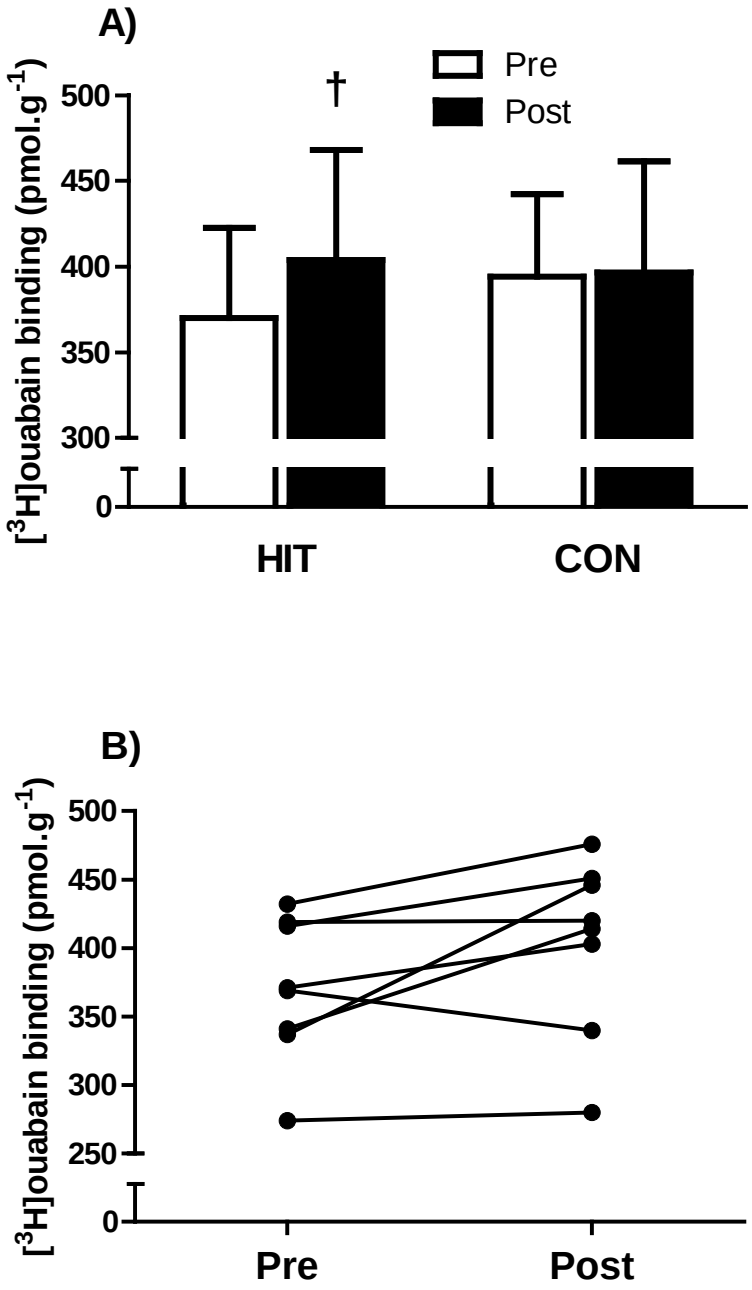


Figure 5.1 Skeletal muscle [^{3}H]ouabain binding site content A) in both training and control groups before and following 12 weeks of HIT and B) individual [^{3}H]ouabain binding site content responses to training. Open bars denote pre, closed bars denote post. Data is presented as mean ± SD.

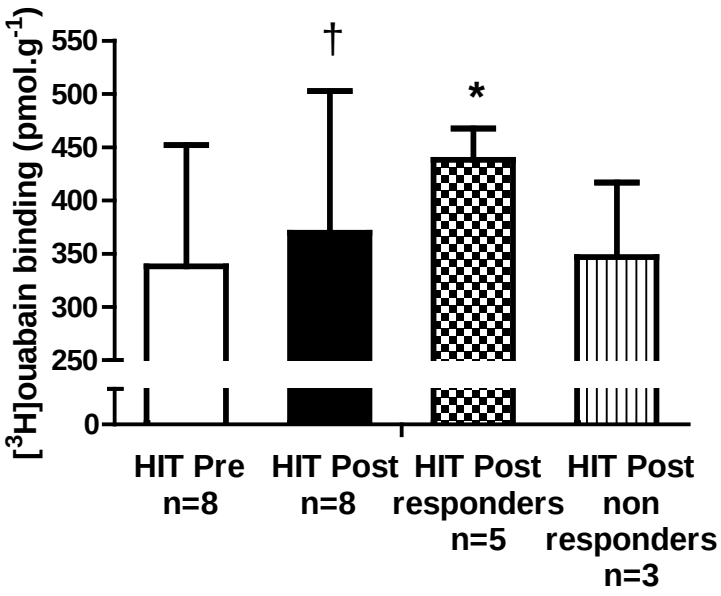


Figure 5.2 [^{3}H]ouabain binding from older adults who completed HIT training. Data is shown for HIT Pre (open bar), HIT post (closed bar) and also Post training separately for the 5 participants who responded to training determined by an increase in [^{3}H]ouabain binding (patterned bar) and for 3 participants who did not respond to HIT (striped bar) * greater than Pre, $p<0.05$, † tendency to be greater than Pre $p=0.07$. Data presented as mean ± SD.

5.4 DISCUSSION

Analysis of NKA isoforms in single fibres revealed that 12 weeks of HIT training in older adults induced fibre-type specific upregulation of, with increased α_2 abundance in type II fibres and β_1 in type I fibres. This demonstrates for the first time that NKA in elderly humans responds to high-intensity interval training in a fibre type specific manner. Importantly when analysed in whole muscle homogenate there were no increases detected in the abundance of α_2 or β_2 isoforms following training, indicating the greater sensitivity of the single fibre method. Training also tended to increase [^{3}H]ouabain binding from all 8 participants; however, when muscle from the five responders out of eight was analysed [^{3}H]ouabain was increased with training, coinciding with the increased abundance of α_2 in type II fibres. The efficacy of the HIT aerobic training was evidenced by increases in VO_2 peak and increase in skeletal muscle mitochondrial protein COX IV in whole muscle homogenate.

5.4.1 NKA isoform and whole muscle changes with HIT in the elderly

This study demonstrates an upregulation of the NKA α_2 and β_2 isoforms in skeletal muscle after HIT in older adults. The α_2 is of high importance during exercise and muscle contraction (He *et al.*, 2001; Radzyukevich *et al.*, 2013) and is the most abundant of the α subunit isoforms comprising approximately 80-85% (Clausen, 2003b). The upregulation of α_2 following training implies a greater synthesis of these isoforms is required to cope with the demands of HIT in type II fibres, possibly due to an increased recruitment of these fibres with HIT than occurs during regular physical activities. It may also be possible that the training may decrease α_2 catabolism. A common finding following training is an increase in the β_1 isoform, but this not found in the current study and mechanisms underlying this lack of increase are not known. The β_1 was shown to be increased with training in old rats (Ng *et al.*, 2003) and has sometimes been reported to be upregulated after training young adults and other times there has been no reported increase (Aughey *et al.*, 2007; Iaia *et al.*, 2008;

Thomassen *et al.*, 2010; Gunnarsson & Bangsbo, 2012). Perhaps the volume of training may be an important factor for the upregulation of β_1 and given in the current study the use of short volume training in this study may have played a role in the lack of responsiveness shown by the β_1 isoform. The β_2 was found to be lower in older adults compared to young in Chapter 4 and perhaps training was able to restore this apparent decrease with age. This suggests that the abundance of β_2 in human skeletal muscle may be important for skeletal muscle function. Both the α_2 and β_2 isoforms were increased in a fibre-type specific manner following no more than 12 hrs of training over a 12 week period in an aged population, demonstrating that the NKA remains highly adaptable in an ageing cohort. Older adults who have been active for 10-12 years had an increased abundance of NKA content by [^{3}H]ouabain binding than inactive older adults (Klitgaard & Clausen, 1989). However this was a cross sectional study and no studies have studied the effects of high-intensity, short duration training on NKA content in a healthy elderly population prior to the current study. Following 12 weeks of HIT in the elderly, [^{3}H]ouabain binding site tended to increase ($p<0.07$). From this cohort it appeared that only five from eight participants who completed training responded; analysis of just those participants revealed an increased [^{3}H]ouabain binding site content compared to pre-training ($p<0.05$), with the non-responders showing no difference in [^{3}H]ouabain binding. Thus the tendency for [^{3}H]ouabain binding to increase following HIT coincided with an increase of the relative abundance of the α_2 isoform exclusively in type II fibres. Numerous studies investigating the effects of training, ageing, injury and disease on NKA content have shown the changes in relative protein abundance of α_2 measured in muscle homogenates, are greater than the changes in [^{3}H]ouabain binding site content (Clausen, 2013b). Given that the measure of α_2 abundance in this thesis was unchanged in whole muscle homogenate and was only upregulated in type II fibres after HIT, the tendency for [^{3}H]ouabain binding to increase may suggest it is possible that the [^{3}H]ouabain binding assay

was not sensitive enough to detect changes that were restricted to only one fibre-type. Similar increases in the relative abundance of NKA α_2 isoform and [^{3}H]ouabain binding has only been reported to occur five times in the literature (Clausen, 2013b). The α_1 and α_3 isoforms have the same ouabain affinity as the α_2 in human skeletal muscle (Wang *et al.,* 2001) and thus all three are measured during the [^{3}H]ouabain binding assay in human muscle. The α_2 is the key NKA isoform associated with increases in [^{3}H]ouabain binding and improvements in exercise performance, with studies in mice showing the α_2 to be the most abundant NKA isoform and to play a large role in muscle contraction and fatigue (Radzyukevich *et al.*, 2013).

This study also found a 48% decrease in the β_3 isoform in type II fibres following training. This was in conjunction with an increase in β_3 abundance seen in Chapter 4. In a training study in rats, the β_3 abundance was reported to decrease by 33% following training in aged rats (Ng *et al.,* 2003). To explain this, it was suggested it may be possible that an elevated abundance of β_3 in skeletal muscle may not be a favourable outcome (Ng *et al.,* 2003). Given the results in the current study are similar to Ng *et al.,* (2003), this suggestion may also be true for humans but this is purely speculative given that only one study has measured β_3 in muscle from aged rats and two human studies have differing results for the β_3 isoform. It is important that further work is conducted to determine the functional implications of alterations to the relative abundance of the NKA β_3 isoform in skeletal muscle with ageing and training.

A limitation of the single fibre blotting technique in this study was the lack of “pre” type II fibres analysed. The strength findings of this study may be enhanced or reduced by the inclusion of more “pre” type II fibres.

5.4.2 Enhanced VO_2 peak and exercise performance following HIT

Training elicited improvements in VO_2 peak which has positive outcomes for older adults, given that aerobic power is a strong indicator for mortality (Blair *et al.*, 1996; Smart, 2013; Weston *et al.*, 2013). The findings from other young and clinical studies also show an ability to improve aerobic power following HIT; thus the current results were somewhat expected (Wisloff *et al.*, 2007; Gibala *et al.*, 2012; Tjønna *et al.*, 2013). However, previous studies in elderly cardiac patients have found much more pronounced improvements than in the current study (Wisloff *et al.*, 2007). This is likely to be attributed to the population in the current study being healthy and accustomed to exercise of some nature; it may be possible that if a less active population was used for the current study a greater magnitude of change would have been detected.

While the exercise was highly intensive in nature, it may be possible that the participants in this study could tolerate higher intensity training had they, like healthy young adults, been pushed beyond 100% VO_2 peak. During exercise training, the average RPE was only 13 out of 20 which corresponds to “somewhat hard” on the Borg scale (Borg, 1982). An assessment of training progress including another incremental exercise test at 6 weeks into the program may have provided a new target HR and further increase in work which may have enabled inducement of greater improvements in fitness than seen in this study. But when training elderly populations an important consideration was the risk of an adverse event and whether the risk of increasing training load would outweigh the training benefits in this population is unclear. However, it is possible that some participants would have tolerated a higher intensity particularly those who were active and this is an avenue for future research.

5.5 CONCLUSIONS

This study demonstrates the fibre-type specific adaptability of several NKA isoforms to high-intensity interval training in older adults and that the use of single fibre western blotting as a sensitive method to detect these changes. High-intensity interval training in older adults increased NKA α_2 isoform in type II fibres and the β_1 isoform in type I fibres which corresponded with an increase in [^{3}H]ouabain binding in five from eight participants. Whilst HIT in the elderly may provide a small risk to participants unfamiliar with intense exercise, once familiarised, HIT was well tolerated amongst older adults and may prove to be an effective exercise modality for improvement of cardiovascular fitness and exercise performance in older adults.

CHAPTER 6 The effects of 23 days of unilateral lower limb suspension (ULLS) on Na^+, K^+-ATPase isoform abundance in single skeletal muscle fibres from healthy young adults

6.1 INTRODUCTION

Muscle function is highly dependent on the maintenance of skeletal muscle cell excitability which is critically dependent on the Na^+, K^+-ATPase (NKA) (Clausen 2003). The NKA family of proteins comprises six isoforms expressed in skeletal muscle each with a unique genetic code and presumably its own function, however the functions for each of the isoforms remain to be fully determined (Blanco & Mercer 1998). It is known that all six isoforms are expressed within the same fibre (Chapter 3). Given the isoforms may have differing roles it is plausible that alterations to their abundance could modulate NKA activity and thereby affect muscle excitability and muscle function.

Skeletal muscle is highly adaptable with increased physical activity (Röckl *et al.*, 2008). The [^{3}H]ouabain binding site content in muscle reflects the NKA content and was higher in individuals who had a greater number of weekly training hours compared to those with less hours of weekly training (Murphy *et al.*, 2007). Individuals who are inactive might then be expected to have lower muscle NKA content than physically active people, but this is unknown and little is also known about changes in NKA isoforms following inactivity. Two studies in humans have reported the effects of injury, which incorporates a large component of inactivity, on NKA content in muscle. Patients with shoulder impingement had a 26% decrease in [^{3}H]ouabain binding in the deltoid muscle of the injured compared to the uninjured shoulder (Leivseth & Reikeras 1994), whereas complete spinal cord injury (SCI) patients had ~40-50% decrease in muscle NKA content compared to able bodied controls (Ditor *et al.*, 2004; Boon *et al.*, 2012). Following long-term SCI, NKA isoforms in vastus

lateralis muscle were markedly reduced, by 75% for α_1, 51% for α_2 and 38% for β_1 (Boon *et al.*, 2012). However, SCI is a severe injury and includes the effects of many factors in addition to inactivity, such as a loss of neurotrophic factors.

Physical inactivity per se has been studied using a unilateral lower limb suspension model (ULLS), which has marked adverse effects on skeletal muscle strength and function and includes reductions in muscle fibre size (Berg *et al.*, 1991; Hackney & Ploutz-Snyder, 2012). No study has investigated the effects of short-term physical inactivity on NKA content and isoform abundance. Muscle is heterogeneous with respect to fibre type composition, but whether the NKA isoforms may be down-regulated in fibre-type specific manner following inactivity is unknown. Therefore the first aim of this study was to investigate the fibre-type adaptability of the NKA isoforms in single skeletal muscle fibres following 23 days of ULLS.

Resistance training (RT) has beneficial outcomes for targeting sarcopenia in older adults (Hurley *et al.*, 2011; Leenders *et al.*, 2013) and retraining atrophied muscle is a critical component of rehabilitation following periods of inactivity where there is a large loss of strength and muscle function, such as recovering from injury and/or casting (Lepley & Palmieri-Smith, 2013). The muscle [^{3}H]ouabain binding site content was increased following RT (Green *et al.*, 1999; Medbo *et al.*, 2001) but the effects on NKA isoforms are unknown. Therefore the second aim of this study was to investigate whether 4 weeks of RT would reverse any reductions in NKA isoform abundance that might occur in single fibres following 23 days of ULLS.

Given that the major NKA isoforms the α_1, α_2 and β_1 isoforms are upregulated by exercise training (Mohr *et al.*, 2007; Green *et al.*, 2008; Benziane *et al.*, 2011) and reduced by chronic SCI (Boon *et al.*, 2012), it was hypothesised that 23 days of ULLS would decrease the abundance of the α_1, α_2 and β_1 isoforms in human skeletal muscle, and based from results

from chapters 3, 4 and 5 in this thesis, it was hypothesised that this would occur in a fibre-type specific manner. Given that the α_3 and β_2 isoforms were unchanged following SCI, it was hypothesised that these isoforms would also be unaltered by ULLS in either fibre-type. Further, it was hypothesised that following resistance training, any reduction in NKA isoforms caused by ULLS would be restored, regardless of fibre-type.

6.2 METHODS

6.2.1 Context

This study was part of a broader study investigating the effects of ULLS on skeletal muscle function, exercise performance, plasma K^+ regulation during exercise and whole muscle [^{3}H]ouabain binding site content and isoform abundance in whole muscle homogenate (Perry, unpublished, 2014).

6.2.2 Participants

Six sedentary healthy adults (4 males, 2 females), gave written informed consent and participated in this study, which was approved by the Victoria University Human Research Ethics Committee and conformed to the Declaration of Helsinki. The physical characteristics of the participants were; age: 22.4± 2.1 years; mass: 71.3 ± 14.3 kg; height: 175.0 ± 11.7 cm; BMI: 23.2 ± 3.6 kg.m^{-2}; VO_2 peak 45.5 ± 5.8 ml.kg^{-1}.min^{-1}; mean ± SD). Participants were excluded if they were using the oral contraceptives pill, had any allergies to aspirin, had a previous or family history of deep vein thrombosis (DVT), blood clotting problems/disorders, or were obese.

6.2.3 Inactivity protocol

The ULLS protocol was based on an earlier described protocol (Berg *et al.*, 1991; Tesch *et al.*, 2004). Briefly, participants were asked to undertake all movements for 23 consecutive days with only the assistance of crutches; where a single leg (left leg) was subjected to

unloading. To minimise the risk of participants developing a DVT, the right shoe was elevated by a 10 cm sole, thus enabling the left leg to hang freely during the 23 days. Twice per week throughout the ULLS period, an antecubital venous blood sample was taken and analysed for D-dimer as a pre-clinical marker of DVT, by an independent clinical pathology lab (Melbourne Pathology).

6.2.4 Re-training protocol

Following the inactivity protocol, participants completed 12 sessions of supervised resistance training focused on the lower limbs, conducted three days per week over four weeks. Participants completed knee extension, leg press, knee flexion, lunge and calf raise exercises, with participants performing three sets of 10-12 repetitions in each session. To apply progressive overload, the resistance lifted was increased once participants could successfully complete three sets and twelve repetitions of an exercise.

6.2.5 Muscle biopsy, single fibre dissection and western blotting procedures

A resting muscle biopsy was taken from the unloaded leg under resting conditions prior to (CON), after ULLS (ULLS) and following the four weeks of resistance training (RT). The biopsy procedure, subsequent single fibre separation from fresh muscle and western blotting technique was as described (Chapter 3). The antibodies were identical to those described previously (Chapter 4, Table 4.1). Due to difficulties in detecting β_3 in this study (possibly due to using a different batch of the antibody), the NKA β_3 isoform was not analysed.

6.2.5.1 Normalisation of single fibres

In this chapter the normalisation of the single fibres from this study had been advanced since the data analysis of chapters 3, 4 and 5; thus, a slightly different and further developed method was undertaken here instead of normalising to total protein. Each fibre was assigned a value for the total protein based on the linear regression equation of the total protein on the

calibration curve (example in figure 6.1, diamond). Following this, the isoform of interest was then assigned a value based on the linear regression equation for the respective isoform on the calibration curve (example in Figure 6.1, square). The relative abundance of each isoform in arbitrary units was then obtained by normalising the value for the isoform over the value obtained over the total protein.

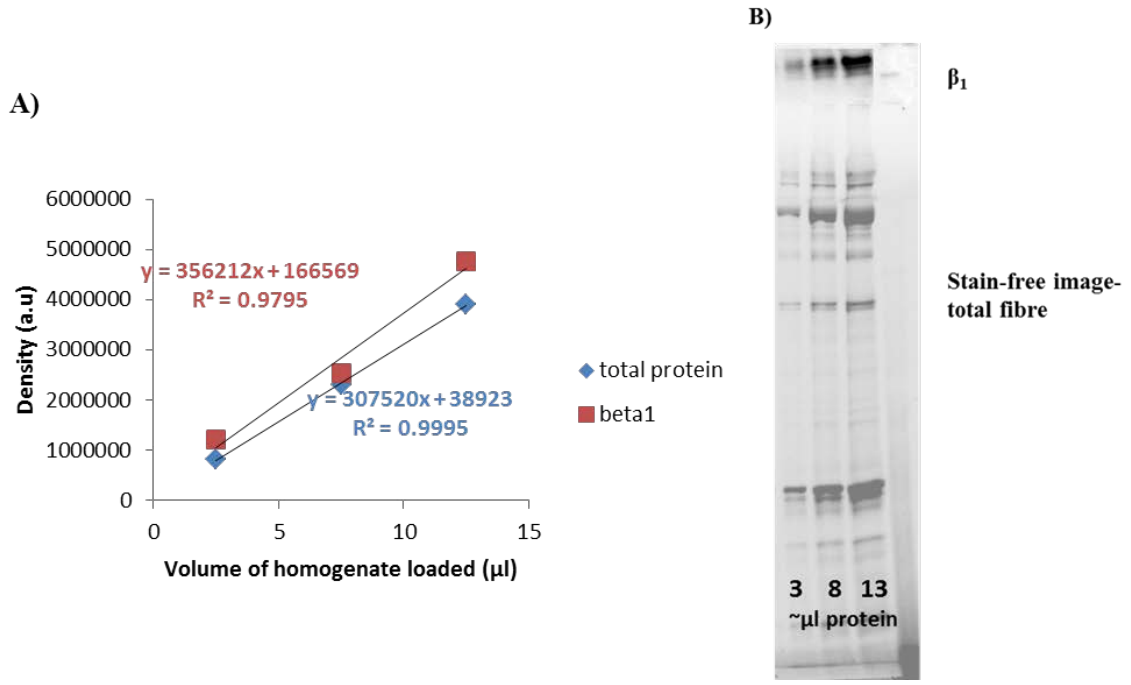


Figure 6.1. A) An example of calibration curves obtained from a gel (total protein, blue diamonds) and a western blot (β_1 isoform, red boxes). Linear regression equations (y = mx + c) shown for each curve. Thereafter, the density for each fibre on the same gel / western was expressed relative to the regression equation. B) Representative blot of the calibration curve for the β_1 isoform and total protein of the whole fibre.

The use of a calibration curve ensures that any non-linearity in the signal intensity (i.e. density, y-axis, Fig 6.1) and amount of sample loaded (x-axis, Fig 6.1) is taken into consideration and is more accurate then normalising each fibre against the total protein in each lane. This is why the use of a calibration curve for total protein in addition to the protein of interest is also important. The use of the same muscle homogenate in preparing a calibration curve across all the gels in the study, and expressing each fibre relative to a point on the standard curve allows all fibres from each gel to be compared against each other. The normalisation of protein abundance for each single fibre to total protein and a point in the standard curve is critical for two reasons. First, the mass of each fibre is unknown and therefore normalisation to total protein is necessary to be able to account for this. Second, in order for fibres to be compared across different gels they must be normalised to a known point, which is the same sample in every gel.

6.2.6 Statistical Analysis

All data is expressed as mean ± SD. All data was log-transformed and analysed using a univariate nested model (linear mixed model) with fibre type as fixed factor and participant as random factor, to detect any differences in NKA abundance from ULLS and following resistance training in both type I and II fibres, and taking into account fibres from the same individuals. Statistical significance was accepted at $p < 0.05$. All statistics were run using IBM SPSS software version 20.

6.3 RESULTS

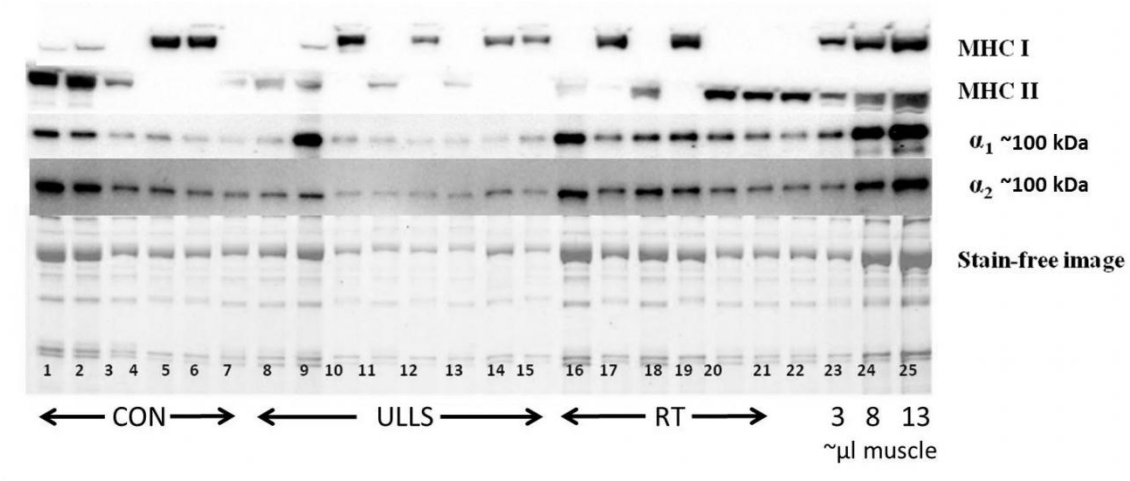
6.3.1 Adaptation of the NKA isoforms to ULLS and following resistance training.

Representative blots of the same samples for α_1 and α_2 are shown in Figure 6.2 and α_3, β_1 and β_2 are shown in Figure 6.3. The number of fibres analysed are shown in Tables 6.1 and 6.2.

The NKA α_1 and α_2 isoform relative abundances were not significantly altered following ULLS and RT in both type I and II fibres compared to CON, although there was a tendency for increased α_1 in type I fibres following ULLS (Figure 6.3, $p=0.09$). The NKA α_3 was decreased in type I fibres after ULLS ($p<0.05$) and remained low following RT ($p<0.05$, Figure 6.3). There was no change in α_3 abundance in type II fibres following ULLS or RT.

The NKA β_1 relative abundance did not change in type I or II fibres following ULLS or RT (Figure 6.3). The β_2 tended to increase in type I fibres following ULLS ($p=0.059$), but following RT there was no significant difference compared to CON. There was no effect of ULLS and RT on the β_2 isoforms in type II fibres.

A)



B)

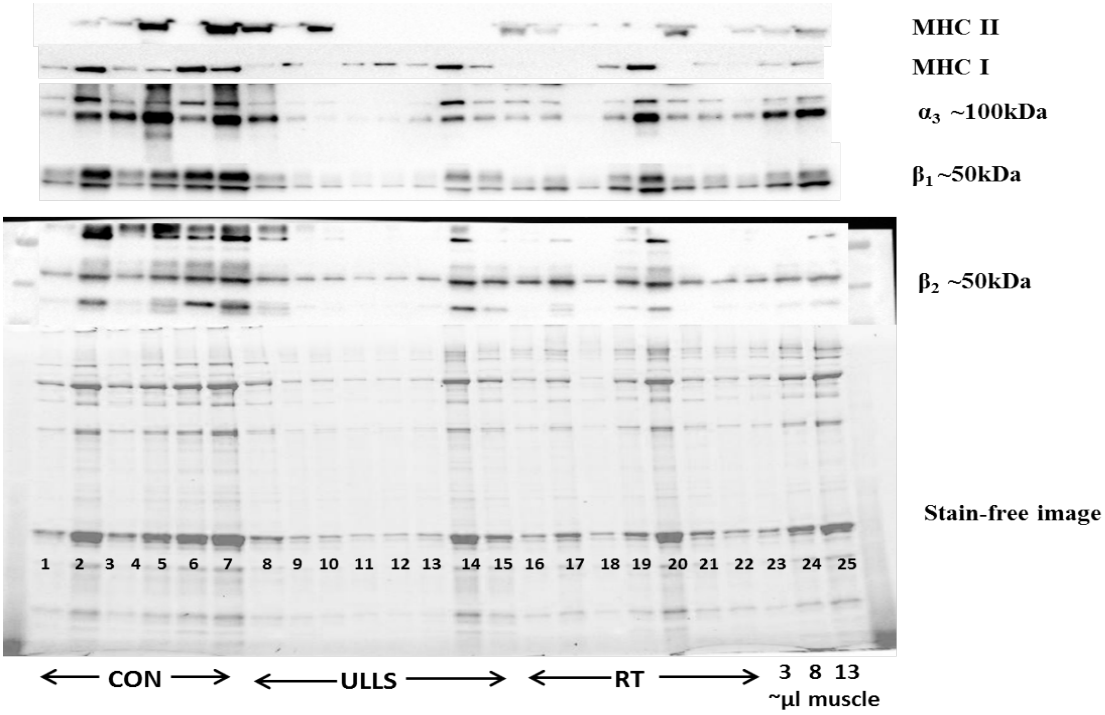


Figure 6.2 Representative blot of a gel run for the A) NKA α_1 and α_2 isoforms at baseline (CON), following ULLS and following 4 weeks of resistance training (RT) from one participant and B) NKA α_3, β_1 and β_2 isoforms at baseline (CON), following ULLS and following 4 weeks of resistance training (RT) from a different participant. Also shown is the

Stain Free image; the density of all the bands in a given lane is used as a measure of total protein in a given lane. A single fibre segment was loaded into each lane and was classified as either a type I or type II fibre based on the MHC I or II isoform presence, respectively (top two blots). Indicated are Lanes 1-7, CON; lanes 8-15, ULLS; and lanes 16-22, RT. Lanes 23-25 represent the calibration curve made up of whole muscle homogenate.

Table 6.1 Number of fibres and participants included in final analysis of type I fibres for each NKA isoform at each sample point.

Type I	**CON**	**ULLS**	**RT**
α_1	N= 4, n=9	N=5, n=16	N=3, n=6
α_2	N= 5, n=10	N=5, n=14	N=4, n=7
α_3	N=3, n=9	N=4, n=11	N=4, n=7
β_1	N=5, n=10	N=5, n=17	N=4, n=9
β_2	N=4, n=6	N=2, n=8	N=4, n=6

N= number of subjects, n=number of fibres included in final analysis

Table 6.2 Number of fibres and participants included in final analysis in type II fibres for each NKA isoform at each time point

Type II	**CON**	**ULLS**	**RT**
α_1	N=4, n=8	N=5, n=11	N=3, n=9
α_2	N=5, n=11	N=5, n=10	N=4, n=11
α_3	N=3, n=7	N=4, n=12	N=3, n=7
β_1	N=5, n=10	N=5, n=11	N=4, n=11
β_2	N=3, n=7	N=4, n=7	N=4, n=10

N= number of subjects, n=number of fibres included in final analysis

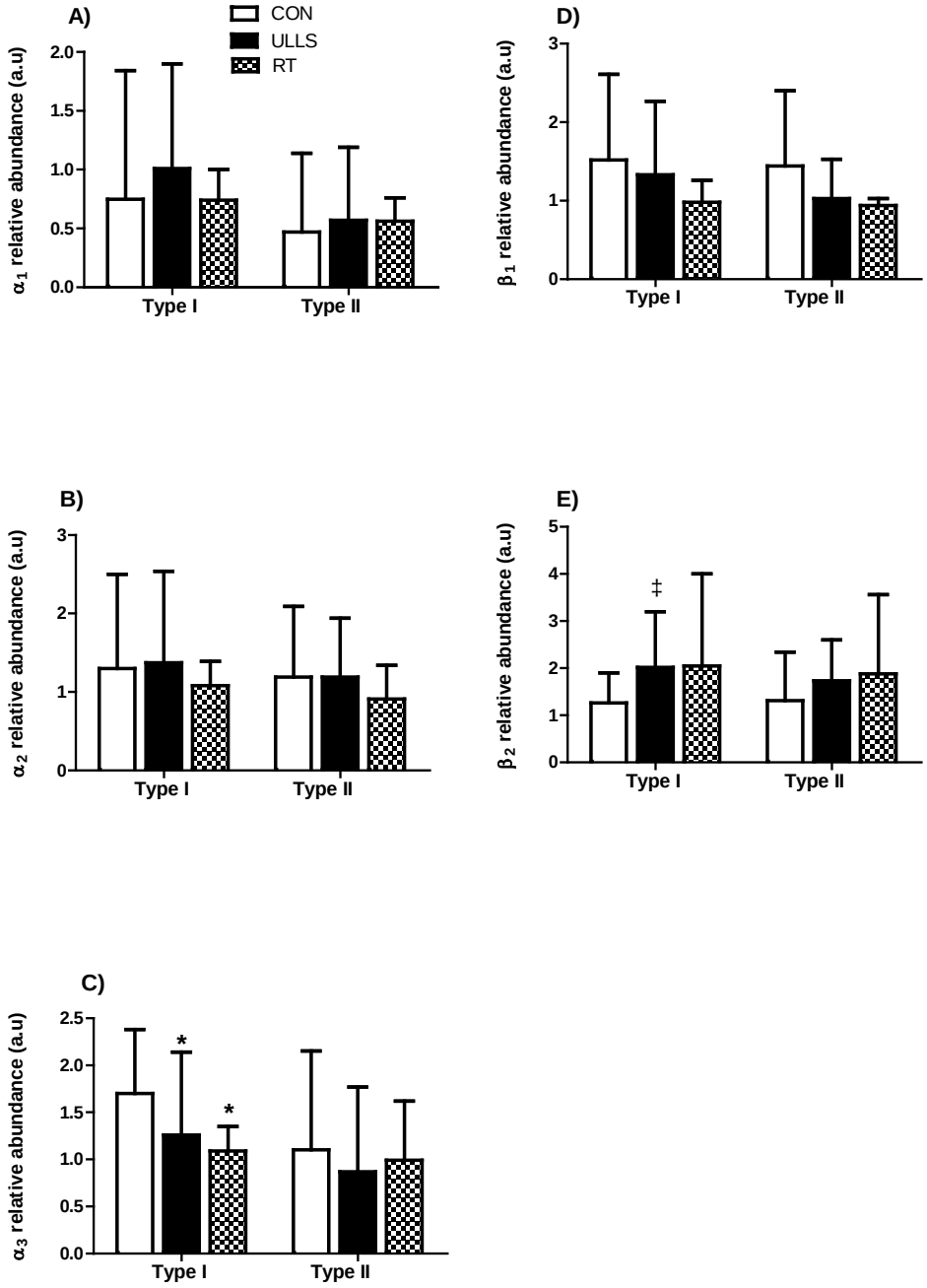


Figure 6.3 Human skeletal muscle Na^+, K^+-ATPase A) α_1, B) α_2, C) α_3, D β_1, and E) β_2 isoforms in type I and II single fibres before (CON, open bars), following 23 days of ULLS (filled bars) and 4 weeks of resistance training (RT, patterned bars). For each fibre, a given isoform was expressed relative to the linear regression curve obtained for that isoform from

the calibration curve on the same gel. * $p<0.05$ different from CON, † $p=0.09$ greater than CON, ‡ $P<0.06$ greater than CON. Data presented as mean ± SD.

6.4 DISCUSSION

This study investigated the effects of ULLS and four subsequent weeks of RT on NKA isoform protein abundances in segments of single human skeletal muscle fibres and yields two novel findings. Firstly this chapter shows that despite 23 days of ULLS, the NKA α_1, α_2 and β_1 isoforms were all maintained without decline in their relative abundances compared with CON. Second, the relative abundances of α_1 and β_2 isoforms tended to increase and α_3 isoform decrease relative to CON and of particular note, these were only seen in type I fibres.

6.4.1 Efficacy of ULLS in loss of muscle strength and mass

The ULLS protocol reduced the muscle's force producing capacity and lean muscle mass with thigh lean mass decreased by ~4.4% as measured by DXA scan, knee extensor strength by ~24%, vertical jump height by ~16% and time to fatigue during cycling decreased ~22.5% (Perry unpublished data 2014). Hence, the ULLS protocol was sufficient to induce declines in muscle strength and function as expected and shown by previous research (Tesch *et al.*, 2004).

6.4.2 ULLS and subsequent resistance training differentially affect NKA isoforms

6.4.2.1 NKA α isoforms

There was a tendency for an increased α_1 relative abundance in type I fibres following ULLS. There was a large variability, reflected by the large SD in the presented data; this is not uncommon in single fibre research, where there has been reported to be as much as a 10-fold variation of HSP72 in single fibres from the same fibre-type from the same participant (Frankenberg *et al.*, 2013). Further, the low sample size is likely to have contributed to the variability (further discussed in 7.1.1). The lack of significant difference may the due to the

statistics run in this study, which took into account the average of each isoform for each fibre-type (further discussed in 7.1.1)

The tendency to upregulated α_1 following ULLS compared to CON, might be a compensatory change to preserve the NKA activity during inactivity, but this remains to be determined. In patients with complete SCI, the α_1 was decreased (Boon *et al.*, 2012) and given the detrimental effects of ULLS on skeletal muscle function and strength, it is surprising that the α_1 was unchanged in the current study, given the sensitivity of the single fibre technique as shown in Chapters 3-5.

Additionally, the lack of alteration in α_2 abundance by ULLS is also surprising given its key role in muscle contraction (Radzyukevich *et al.*, 2013) . In guinea pig hindlimb muscles, 21 days immobilization decreased [^{3}H]ouabain binding by ~25% (Leivseth *et al.*, 1992) and in the guinea pig the α_2 is the only isoform detected by [^{3}H]ouabain binding. In human vastus lateralis muscle taken from patients with chronic SCI, the α_2 abundance measured in a fractioned muscle homogenate was reduced by 51%; this also reflected the decline in [^{3}H]ouabain binding site content from ~220 to ~100 pmol.g wet weight^{-1} (Boon *et al.*, 2012). Interestingly, in that study, a cohort of patients who suffered incomplete cervical SCI for 12 months had a similar α_1, α_2 or β_1 isoform abundance compared to able bodied controls when their muscle was assessed within 12 months of injury. It was believed that neuromuscular activity of incomplete SCI patients influenced the outcome of these results (Boon *et al.*, 2012). Therefore it may appear that the ULLS protocol employed may not be sufficient to elicit changes in the catalytic α_1 or α_2 isoforms. This suggests that these key isoforms are preserved despite declines in muscle size, strength and function.

An interesting finding was that the α_3 abundance was decreased after ULLS, exclusively in type I fibres. Little is understood about the role of α_3 in skeletal muscle and results from this

study contrast findings in fractionated whole muscle from SCI patients, where the α_3 was not decreased after SCI (Boon *et al.*, 2012). Methodological differences and injury versus inactivity differences per se make the two studies difficult to compare (see 6.4.3). The functional implication of a decrease in α_3 in type I fibres following ULLS may be an initial response to short term inactivity, given the trend for α_1 to be upregulated and whilst there is synthesis of new α_1 isoforms, which may be required for increased NKA activity (discussed below), the α_3 remains decreased. Further, the α_3 was not restored to CON levels following RT, suggesting that four weeks of RT was not adequate stimulus for α_3 to be restored. Of note, knee extensor strength was restored by participants in this study (Perry unpublished data 2014) and this may suggest the α_3 plays a role in skeletal muscle not related to muscle contraction.

6.4.2.4 NKA β isoforms

There was no change in the relative abundance of the NKA β_1 isoform in either type I or II fibres, whilst the β_2 tended to increase following the 23days of ULLS in type I fibres ($p=0.07$) and was not changed following RT. In the whole muscle samples from the same study there was also no change in the abundance of the β_1 and a tendency for β_2 to be increased (Perry unpublished 2014). Following complete SCI type there was a decrease of the β_1 (38%) but no change was seen with the β_2 isoforms (Boon *et al.*, 2012). It may be possible that the tendency for the α_1 and β_2 isoforms to be increased after 23 days of ULLS perhaps due to a decline in NKA activity and under basal conditions these isoforms have begun to increase to compensate for decreased NKA activity. Why the alterations have occurred in type I fibres are unknown. Few studies have investigated the abundance of the β isoforms following injury and this study, whilst opposing findings to Boon *et al.*, (2012) does not confirm adaptability of the β isoforms but tendencies may suggest the β isoforms need to be upregulated to regulate NKA activity during inactivity.

6.4.3 Comparisons to and limitations in previous studies

There are four key difficulties in trying to compare the study by Boon *et al.,* (2012), against finding from this study. Firstly, the chronic CSI model is a severe injury model in respect to both the duration of the injury and type of "inactivity" imposed by the injury to the participants. Secondly, there are complications from injury such as altered endocrine homeostasis, use of prescribed medications and inflammatory responses, which may affect NKA isoform abundances and total NKA content. It was reported by Boon *et al.,* (2012) that these factors were unlikely to affect NKA content or isoform abundance in their study. Thirdly, only a proportion of the muscle protein was analysed in the fractionated tissue, although the protein recovery was reported; they assumed that the recovery would be the same in all samples which may not be the case after SCI. Fourthly, Boon *et al.,* (2012) normalised the density of the NKA isoforms on their western blots to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which may make the interpretation of these results problematic. In Chapter 4 it was found that GAPDH was more abundant in type II fibres and compared to type I and fibre-typing analysis from skeletal muscle biopsies from chronic SCI patients within the first two years of SCI expressed a large proportion of MHC IIb (63%) and IIa (33%) isoform expression compared to MHC I (< 5%) (Ditor *et al.*, 2004). Any change in fibre type distributions, as seems apparent with SCI, suggests that GAPDH would also be altered between SCI patients and controls. Hence without data showing the effect of SCI on GAPDH protein content, then it is difficult to interpret the data from Boon *et al.,* (2012).

6.4.4 Limitations in the current study

There are a number of limitations of this study. Firstly, the FXYD1 (phospholemman) is an important known regulator of NKA, which when phosphorylated binds to the α subunit and increases Na^+ affinity of the NKA (Bilbert *et al.,* 2008). However, the current study did not measure either total FXYD1 or FXYD1 phosphorylation sites. Secondly, the activity of total

NKA was not determined and so it cannot be presumed that any change in NKA isoform reflects the activity of the heterodimer complex. The findings presented of fibre-type specific adaptability of NKA isoforms, do suggest that any NKA activity measurements should be conducted in specific fibre types.

Thirdly, this study is limited by a sample size of 6 subjects. Recruitment was difficult and occurred over a two year period. Regardless this chapter still highlights the importance of the single fibre approach in measuring NKA isoforms; given all changes seen in the present study were exclusively in type I fibres. In whole muscle homogenates in this study, there was no detection of any change in NKA isoforms, except for a tendency for β_2 to be upregulated (Perry unpublished data 2014), while no detection of any change in the α_3 or tendencies for the α_1. The decrease in α_3 was not detectable in whole muscle, and tendencies for the increase in α_1 were not detected, likely due to the use of mixed fibre muscle sample being sufficiently sensitive enough to identify small trends that were detectable, but not significant within single fibres. Other unloading studies have found greater atrophy in type I fibres in both human and rodent. It has been identified the human soleus muscle is comprised of 84% type I and only 16% type II fibres (Trappe *et al.*, 2001). In addition the soleus is heavily recruited during walking and therefore the combination of a heavily recruited muscle primarily comprised of type I fibres may cause a greater detrimental effect in the soleus muscle compared to vastus lateralis in 23 days. It is also possible that the single fibre analysis was underpowered and that the collection of more fibres, may have seen the α_1 and β_2 trends become significant. Future research should consider examining the soleus and vastus lateralis to establish differences which may occur following 23 days of unloading.

6.5 CONCLUSIONS

This study shows the preservation of the NKA α_1, α_2 and β_1 isoforms to short-term unloading, with only a decrease of α_3 in type I fibres observed. If a longer duration of ULLS had been employed it may have been possible to elicit changes to the key NKA α_2 and β_1 isoforms. Nonetheless, negative effects of unloading were shown on the NKA α_3 isoforms and show a tendency to upregulate α_1 and β_2. Whether these changes with ULLS effects muscle excitability and NKA function remains to be determined.

CHAPTER 7 GENERAL DISCUSSIONS, CONCLUSIONS AND DIRECTION FOR FUTURE RESEARCH

7.1 GENERAL DISCUSSION

This thesis investigated the relative abundance of the skeletal muscle NKA isoforms in human skeletal muscle single fibres and their adaptability following four different conditions, including repeated-sprint exercise training in young adults (Chapter 3), ageing (Chapter 4), following high-intensity interval training in the elderly (Chapter 5) and short-term inactivity via the use of unilateral lower limb suspension in young adults (Chapter 6).

7.1.1 The expression of the skeletal muscle NKA isoforms in single muscle fibres

A key initial finding of this thesis was that the all six of the skeletal muscle NKA isoforms expressed in human skeletal muscle were found in both type I and II fibres and that all six were all co-expressed in each fibre. The measurement of all six isoforms in one single muscle cell was possible by placing a single fibre in 10 µl of SDS dividing into two aliquots and running 5 µl from the 10 µl in one gel, the other 5 µl in a separate gel; thereby running one single fibre across two gels.

The findings regarding the relative abundance of the NKA α_1, α_2, β_1 and β_3 isoforms in different fibres were consistent and showed that in fibres obtained under resting conditions each of these isoforms displayed a similar abundance in both type I and II fibres. A summary from each of these isoforms across the four studies is shown in Tables 7.1-7.4.

Table 7.1 Comparison of findings using two statistical approaches to assess NKA α_1 isoform abundance in type I and II fibres in healthy young people, in samples collected at baseline prior to each intervention in each study.

Chapter	Single fibre normalisation	N	Fibre Type		Statistical Analysis	
			Type I	Type II	Univariate	*t*-test
3. Characterisation of isoforms in single fibres	Average of type II fibres	17	0.95±0.45	0.97± 0.63	NS	NS
3. RSE training	Calibration curve	8	1.09±0.86	0.93±0.59	NS	NS
6. ULLS	Calibration curve	6	0.46±0.73	0.47±0.67	NS	NS
4. Young vs. Old (Young only)	Calibration curve	9	0.96±0.77	0.88±0.81	NS	NS

Table 7.2 Comparison of findings using two statistical approaches to assess NKA α_2 isoform abundance in type I and II fibres in healthy young people, in samples collected at baseline prior to each intervention in each study.

Chapter	Single fibre normalisation	N	Fibre Type		Statistical Analysis	
			Type I	Type II	Univariate	*t*-test
3 Characterisation of isoforms in single fibres	Average of type II pre fibres	17	1.03±0.42	0.97±0.35	NS	NS
3. RSE training	Calibration curve	8	1.07±0.60	1.07±0.48	NS	NS
6. ULLS	Calibration curve	6	0.93±0.34	1.02±0.76	NS	NS
4. Young vs. Old (Young only)	Calibration curve	9	0.72±0.30	0.75±0.24	NS	NS

Table 7.3 Comparison of findings using two statistical approaches to assess NKA β_1 isoform abundance in type I and II fibres in healthy young people, in samples collected at baseline prior to each intervention in each study.

			Fibre Type		Statistical Analysis	
Chapter	Single fibre Normalisation	N	Type I	Type II	Univariate	*t*-test
3 Characterisation of isoforms in single fibres	Average of type II pre fibres	17	1.04±0.53	1.06±0.51	NS	NS
3. RSE training	Calibration curve	8	0.59±0.60	0.68±0.40	NS	NS
6. ULLS	Calibration curve	6	1.52±1.09	1.44±0.96	NS	NS
4. Young vs. Old (Young only)	Calibration curve	9	0.87±0.50	0.96±0.58	NS	NS

Table 7.4 Comparison of findings using two statistical approaches to assess NKA β_3 isoform abundance in type I and II fibres in healthy young people, in samples collected at baseline prior to each intervention in each study.

			Fibre Type		Statistical Analysis	
Chapter	Single fibre normalisation	N	Type I	Type II	Univariate	*t*-test
3 Characterisation of isoforms in single fibres	Average of type II pre fibres	17	0.92±0.61	1.11±0.54	NS	NS
3. RSE training	Calibration curve	8	1.34±1.57	1.61±1.22	NS	NS
4. Young vs. Old (Young only)	Calibration curve	9	0.59±0.50	0.52±0.37	NS	NS

For Tables 7.1-7.4 All data is expressed as mean ± SD. All data presented as arbitrary units (a.u.). NS= not significant. N= number of subjects.

However findings regarding the fibre-type specific differences abundance of the NKA α_3 and β_2 isoforms showed some inconsistencies across different chapters, either showing both to be more abundant in type II fibres compared to type I or to be similarly expressed in both type I and II fibres. A summary for the NKA α_3 and β_2 isoforms from each of the four studies are shown in Tables 7.5-7.6. The following section examines the possibility that different statistical approaches undertaken might be responsible for some of the discrepancies between findings in this thesis and other papers.

Table 7.5 Comparison of findings using two statistical approaches to assess NKA α_3 isoform abundance in type I and II fibres in healthy young people, in samples collected at baseline prior to each intervention in each study.

			Fibre Type		Statistical Analysis	
Chapter	Single fibre normalisation	N	Type I	Type II	Univariate	*t*-test
3. Characterisation of isoforms in single fibres	Average of type II pre fibres	17	0.78±0.49	0.94 ± 0.41	p=0.058	p<0.04
3. RSE training	Calibration curve	8	0.59±0.36	0.68±0.40	NS	NS
4. ULLS	Calibration curve	6	1.70±0.68	1.10±1.05	NS	NS
5. Young vs. Old (Young only)	Calibration curve	9	0.76±0.54	1.17±0.72	p<0.05	p<0.05

Table 7.6 Comparison of findings using two statistical approaches to assess NKA β_2 isoform abundance in type I and II fibres in healthy young people, in samples collected at baseline prior to each intervention in each study.

			Fibre Type		Statistical Analysis	
Chapter	Single fibre Normalisation	N	Type I	Type II	Univariate	*t*-test
3. Characterisation of isoforms in single fibres	Average of type II pre fibres	17	0.77±0.52	0.98±0.44	p<0.05	p<0.05
3. RSE training	Calibration curve	8	1.49±0.89	1.36±0.77	NS	NS
4. ULLS	Calibration curve	6	1.26±0.64	1.31±1.03	NS	NS
5. Young vs. Old (Young only)	Calibration curve	9	1.15±0.47	2.11±0.97	p<0.10	p<0.05

Table 7.5-7.6 All data is expressed as mean ± SD. All data presented as arbitrary units (a.u.). NS= not significant. N= number of subjects.

The fibre-type discrepancy of the NKA α_3 and β_2 is highly unlikely to be caused by a methodological error or an issue with using two different normalisation procedures for single fibres, as the α_1, α_2, β_1 and β_3 isoforms showed to be consistently abundant in type I and II fibres from all four studies. However, it is possible these fibre-type inconsistencies may be at least partially attributed to the statistical analysis used. The primary statistical analysis conducted in this thesis was the univariate nested model of analysis, which has the advantage over a traditional independent *t*-test, of taking into account the individual variability of each participant. This is done by taking the mean of all type I fibres from one participant and comparing them to the mean of the type II fibres from that same participant. A fibre-type specific difference in a protein will be determined from the mean from each participant, instead of all of the fibres. This is a critical component of single fibre analyses, as within two different fibres of the same fibre-type taken from the same person, it has been reported that the variation in relative protein abundance can be as large as 10-fold (Frankenburg *et al.*, 2013).

Previous research investigating fibre-type specificity of proteins in single skeletal muscle fibres have primarily used *t*-tests for statistical analysis, where only the characteristics of each fibre and not the participant are taken into consideration (Murphy, 2011; Lamboley *et al.*, 2013; Thomassen *et al.*, 2013). In the instance that the fibre-type specificity of a protein is the only research question addressed then a *t*-test can also be argued to be a suitable way to analyse data. Therefore in this thesis both a univariate nested model of variance and independent *t*-tests was performed in each chapter when determining fibre-type specificity of the NKA isoforms. However, the *t*-test was not used to assess the effectiveness of each condition in altering NKA isoforms, including training in both young and old, ULLS and ageing.

The results from the *t*-test analyses showed the same outcome to the univariate for NKA α_1, α_2, β_1 and β_3 isoforms, with each showing no fibre-type specificity (Tables 7.1-7.4). Results for the α_3 and β_2 isoforms from the *t*-test analyses were different to the univariate analyses, with the *t*-test analysis able to detect significant differences of an increased abundance of the α_3 and β_2 in type II fibres compared to type I; whereas in contrast the univariate was only able to detect a trend (Tables 7.5-7.6).

In the instances for Chapter 3 where only the RSE group was analysed (n=8) and in Chapter 6 with ULLS (n=6) there was no fibre-type specificity for either the α_3 and β_2 isoforms at baseline. This difference in fibre-type specificity compared to different chapters may be caused by two reasons. The first may be due to a small sample size for the number of fibres analysed. It has been found in one study the NKA α_2 isoform was more abundant in type II fibres, with no fibre-type differences detected for the α_1 and β_1 isoforms, from only six participants (Thomassen *et al.*, 2013). Despite Thomassen *et al.*, (2013) having the same number of participants as the ULLS chapter; Thomassen *et al.*, (2013) analysed 48 type I and 88 type II fibres from these six participants under resting conditions; in the ULLS there was a much smaller number of fibres included in final analysis (24 at baseline). This may suggest that in order to effectively and accurately determine any fibre-type specificity of the NKA isoforms, measuring in a large number of fibres is more important than the number of participants. This has also been shown when the fibre-type specificity of AMP-activated protein kinase (AMPK)-α_1 and AMPK-β_1 isoforms were successfully determined in human single fibres collected from biopsies obtained from only three healthy young participants (Murphy, 2011).

A second potential reason for inconsistencies for fibre-type specific adaptations in the expression of the α_3 and β_2 isoforms in this thesis may be due to the variation between

different participants. It is possible that (unlike some metabolic and contractile proteins) whether the NKA isoform abundance is fibre-type specific may be dependent on each individual, in particular the amount of physical activity conducted by participants on a daily basis. If so, this could also explain differences between this thesis and the study by Thomassen *et al.,* (2013) regarding the fibre-type specificity of the α_2 isoform, where Thomassen *et al.,* (2013) reported a ~37% greater abundance if the α_2 in type II compared to type I fibres, whereas in all experimental chapters of this thesis, the α_2 abundance was not different between type I and II fibres. This is despite both studies measuring the α_2 isoform in a relatively equal and large number of type I and II fibres. In most instances both studies used the same primary antibody, and where a different antibody was used, evidence was provided showing the use of a different antibody was unlikely to cause the differences between studies (Chapter 3, Figure 3.3). Finally in Chapter 3, all fibres were normalised to the average of the type II fibres, as done by Thomassen *et al.,* (2013). The univariate analysis did detect subject differences, which suggested that some participants had a greater abundance of α_3 and β_2 but an interaction between fibre-type and participant was not detected, this may have been restricted by a limited participant sample size. Further, making a subject and fibre-type interaction more difficult to detect was that there was not always a type I and II fibre from each participant analysed in chapter 3; thus this would have further reduced the sample size available for this univariate analysis . Thus, in order for future research to avoid these limitations which have occurred in this thesis, for studies where fibre-type specific responses in human are of interest it is highly recommended that a large sample size of participants and a large number of fibres are analysed.

Another important finding similar to the young adults was that older adults also expressed all six NKA isoforms in both type I and II single fibres. There was no fibre-type specificity of any of these isoforms and this outcome was not affected by the type of statistical analysis

conducted (data not shown). The potential functional implications of the fibre-type specificity of these isoforms have already been discussed in each respective thesis chapter and therefore only a brief summary of each of the α β isoform will be included in the general discussion.

7.1.2 Adaptability of the NKA isoforms measured in single muscle fibres

Another key finding of this thesis was that NKA isoforms were adaptable with RSE, ULLS, and HIT in the elderly, and whilst these changes tended to be small and confined to a few isoforms, regardless these adaptions occurred in a fibre-type specific manner.

The NKA α_1 abundance was not increased in either type I or II fibres following RSE in young, or after HIT in the elderly. In muscle homogenates the α_1 has been reported to increase following intensive training including intermittent knee extensor exercise (Nielsen *et al.*, 2004), 2 hours of cycling at 60% VO_2 max for 3 consecutive days (Green *et al.*, 2008) and running via intensive sprint training (Iaia *et al.*, 2008). Other training studies have also shown no change in α_1 abundance but did however induce increases in the relative abundance of the α_2 isoform; these included two weeks of high-intensity training comprising both speed endurance training and small sided soccer drills (Thomassen *et al.*, 2010) and speed endurance training alone (Mohr *et al.*, 2007; Bangsbo *et al.*, 2009). The training stress from the RSE may not have not had provided an adequate training volume, which may have been a contributing factor to a lack of responsiveness seen by the α_1 isoform in this chapter.

Following HIT in the elderly there was an increased abundance of the α_2 isoform, but no change in the α_1. The α_2 isoform has been shown to be increased following intensified training in healthy young adults (Nielsen *et al.*, 2004; Mohr *et al.*, 2007; Green *et al.*, 2008; Bangsbo *et al.*, 2009; Thomassen *et al.*, 2010; Benziane *et al.*, 2011). In this thesis the α_2 abundance was also increased in type II fibres of older adults following HIT. However the NKA α_2 isoform was not affected by RSE training. Whilst the signalling mechanism for the

increase in NKA isoforms is not entirely known, it is believed that multiple signals are required to translate into the synthesis of new NKA isoforms from mRNA (Clausen, 2013b). It is likely that despite the training being of a high-intensity, the short duration of the program meant that this intracellular signalling threshold was not met. Finally, the α_2 abundance was resilient to 23 days of ULLS in both type I and II fibres. This was surprising given that inactivity or injury have shown decreases in [^{3}H]ouabain binding, including following immobilisation in ovine skeletal muscle (Jebens *et al.*, 1995), spinal cord injury in humans (Ditor *et al.*, 2004; Boon *et al.*, 2012) and impingement of the deltoid muscle (Leivseth *et al.*, 1992). Given the resilience to ULLS and tight regulation of the NKA α_2 isoform it may be that the critical role played by the α_2 isoform in the maintenance of skeletal muscle excitability is too important to be easily altered; it therefore may mean that only extreme periods of skeletal muscle disuse can induce large changes in α_2 abundance.

Alternatively the α_3 isoform showed malleability in skeletal muscle and further investigation into its role is warranted to determine the potential functional outcomes of findings from this thesis. The α_3 relative abundance decreased in type I fibres following ULLS. Very few studies have measured the α_3 in human skeletal muscle and therefore comparisons against other research are limited. Other research has shown the NKA α_3 protein abundance to be increased following acute prolonged submaximal exercise (Murphy *et al.*, 2006a), but not after 10 days of training comprising both intense and continuous exercise (Benziane *et al.*, 2011) or after intense interval training in well trained athletes (Aughey *et al.*, 2007). Research in mice has shown the α_3 in skeletal muscle may not be critical in physical performance (Lingrel *et al.*, 2003). Further, in Chapter 6, four weeks of resistance training following ULLS restored muscle strength to baseline; however, the relative abundance of NKA α_3 which was decreased by ULLS remained lowered. The decreases in α_3 following ULLS may

be related to a specific function but given that no measure of NKA activity were made in this thesis and the role of α_3 is relatively unknown, this is unclear.

In this thesis the β_1 was only upregulated with RSE training in type I fibres; others have also been shown the β_1 to be increased in homogenate extracts following a similar type of sprint training (Mohr *et al.,* 2007). Given the β_1 was the sole NKA isoform upregulated following RSE training; it was surprising that the β_1 wasn't altered by any other conditions in this thesis. The β_1 is believed to be an important isoform in skeletal muscle function and perhaps like the α_2 may be well maintained by physical activity and requires a severe bout of disuse to induce a detrimental effect.

The β_2 tended to be increased in type I fibres following ULLS and as per the α_3 isoform, a larger sample size may have detected a significant effect and in Chapter 6 it was speculated this may have had implications for NKA activity. Given there were no measurements of NKA activity, FXYD1 phosphorylation or FXYD1 protein abundance after ULLS, therefore this cannot be confirmed.

The physical activity levels of all participants may have contributed to the extent of the alterations seen in isoform abundance. For example, highly trained adults may have seen greater loss of NKA isoform abundance compared to the recreationally active participants which were recruited. Apposing this, adults with lower physical activity levels may have had larger increases in NKA isoforms. As it stands findings from this thesis are not comparable to other studies as there is currently no research investigating adaptability of the NKA in single fibres following various interventions.

7.1.3 Major NKA isoforms and [^{3}H]ouabain binding site content are unchanged in ageing

The NKA α_2 abundance was unaffected by ageing, which contrasts previous findings in older adults (McKenna *et al.,* 2012). However, normalisation of samples to GAPDH may have

contributed to misinterpretation of results in that study. The physical activity levels of the older adults in this study may have contributed to the relative abundance of α_2 older adults not differing from the young. There was additionally no difference in [^{3}H]ouabain binding site between the young and elderly adults as previously seen in other research (Klitgaard & Clausen, 1989; McKenna *et al.*, 2012). This is consistent with the lack of differences in the relative abundance of NKA α_1 and α_2 isoform abundance between old and young. The lack of [^{3}H]ouabain binding difference was despite decreases in the relative abundance of α_3 in Old compared to Young, consistent with this isoform being of low abundance in skeletal muscle. The young participants in this study were recruited after the all older adults to specifically match the two groups for hours of self-reported physical activity per week, so that weekly hours of exercise could be minimised as an external factor affecting the outcome of results, which has been seen in healthy young people (Murphy *et al.*, 2007). It appears by maintaining normal physical activity the decreases in NKA content may be delayed. The age of the participant is also critical as decreases in NKA content have been seen in older adults aged between 69-81 years compared to 55-68 year olds (Perry *et al.*, 2013) and this was observed in Chapter 4, while increase in [^{3}H]ouabain binding in 65-69 year olds compared to young adults also seen in Chapter 4 is speculated to be related to an increase membrane area of older adults due to a decreased fibre size, in particular of type II fibres (Harrison *et al.*, 1994; McKenna *et al.*, 2012). More work is required to determine this.

The NKA α_3 and β_2 abundances were lower in type II fibres in older adults compared to young, whilst the β_2 in type I also tended to be decreased compared to young. The β_2 appeared to be restored following HIT in the elderly, increasing by 52% in type I fibres, further suggesting that β_2 isoform has an important functional role in skeletal muscle. In this thesis the β_3 isoform only tended to be increased in ageing in type I fibres in contrast to reported decreases in type I fibres following training in the aged (Ng *et al.*, 2003). The

functional implications of this are unknown, but it has been speculated that in the rat a greater β_3 isoform abundance is not preferred in skeletal muscle (Zhang *et al.*, 2006). Given both rat and human skeletal muscle appear to show the same fibre-type expression of the β_3 isoform, as well as similar adaptability with ageing and training, the β_3 function may be similar in rat and human skeletal muscle. To date no mechanistic studies have been conducted as to investigate what the potential functions of the β_3 in skeletal muscle.

7.3 CONCLUSIONS

7.3.1 Study One

This study shows clearly no difference between the major fibre types for the two dominant α isoforms (α_1 and α_2) and for the major β isoform (β_1). The β_2 was found to have a greater abundance in type II fibres, as did the α_3 (when analysed using a *t*-test). The less well understood β_3 isoform also showed no significant fibre-type expression.

RSE training increased the relative abundance of the NKA β_1 isoform in type I fibres, but with no other isoform abundance altered by training.

2.3.2 Study Two

This study demonstrates that fibre-type specific differences of the NKA to ageing were constrained to α_3 and β_2 isoforms, with lower α_3 isoform in type II fibres and lower β_2 isoform in type I fibres. There was no difference found in total NKA content as measured by [^{3}H]ouabain binding site content, unless the individuals were separated around the median age into two distinct groups. Finally, this study shows that the use of GAPDH to normalise NKA isoforms in whole muscle homogenate from ageing populations is not appropriate and may provide misleading results.

7.3.3 Study Three

This study showed that high-intensity interval training in older adults increased the NKA α_2 isoform abundance in type II fibres and the β_1 isoform abundance in type I fibres which corresponded with an increase in [^{3}H]ouabain binding in five from eight participants. There were also improvements in VO_2 peak and peak workrate which may suggest that HIT could be an effective exercise modality for improvement of cardiovascular fitness and exercise performance in older adults.

7.3.4 Study Four

This study shows for the first time NKA isoforms response to short-term unloading in single fibres, but detected only a decrease of α_3 in type I fibres, which was unable to be restored by subsequent resistance training. Additionally there was tendency for the NKA α_1 and β_2 to be upregulated following ULLS. The functional effects of these outcomes on NKA activity remain to be determined.

7.4 FUTURE RESEARCH DIRECTION

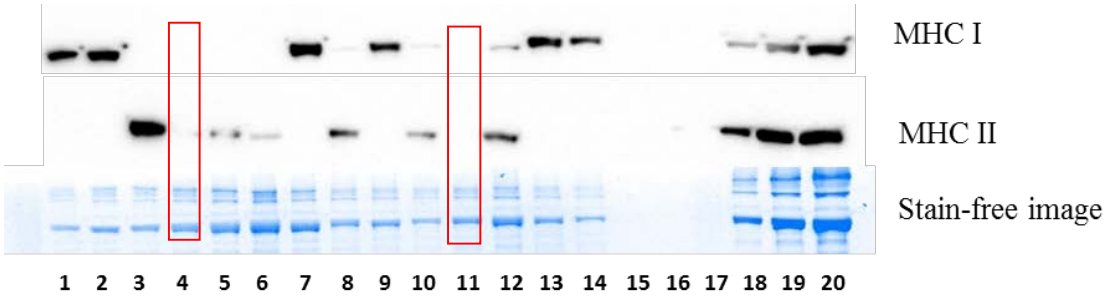
The measurement of NKA activity, FXYD1 abundance and the phosphorylation of FXYD1 following acute and chronic exercise would have provided an insight into the NKA activity and its regulation in this thesis. Problems with the FXYD1 antibody prevented the measurement of FXYD1 relative abundance. Immediately post-exercise biopsies were not obtained from the older adults, as the collection of additional biopsies may have deterred participants from entering into the study and restricted the amount of participants recruited. In future research obtaining post-exercise biopsies from older adults should be considered to allow FXYD1 phosphorylation measurement.

Future research should also investigate whether a longer or different type of training already proven to increase [^{3}H]ouabain binding site content or the relative abundance of NKA α_1 and α_2 isoforms in whole muscle homogenate can induce fibre-type specific upregulation of the α isoforms in young people. Future research in inactivity should investigate whether more sustained ULLS can induced before declines of key NKA isoforms (i.e. α_1,α_2 and β_1). Additionally, with the muscle already obtained from the ULLS study it would be of interest to determine whether the activity of the NKA was affected after 23 days of ULLS, even though there was no effect on NKA content (Perry unpublished 2014).

The use of HIT in the elderly proved a successful tool for the improvement in aerobic power and the upregulation of NKA isoforms in older adults; however, there was a small risk to participants suffering an adverse event with this training. Whether intensity is a critical factor in exercise for healthy older adults or whether a work matched continuous aerobic exercise program would have produced the same results as seen by the HIT is an area for future research. Further advancement of this research could be conducted by including the analysis of hybrid fibres. As we were primarily focused on pure type I and II fibres this analysis was not undertaken. Future research should focus on the adaptability of NKA isoforms in hybrid fibres in addition to type I and II fibres.

One limitation of this thesis is that antibodies used for MHC analysis in the western blotting technique could only detect type I and II single fibres, whilst the pure IIx antibody is only able to detect type IIx fibres when conducting immunohistochemistry analysis. The antibody used to detect MHC II in this thesis is reported to be able to detect MHC II/x fibres in human skeletal muscle, however, throughout experiments conducted throughout this thesis there were some fibres analysed where neither the MHC II nor MHC I isoform could be detected but a fibre was present in the lane; this was believed to be a pure type IIx fibre (Figure 7.1).

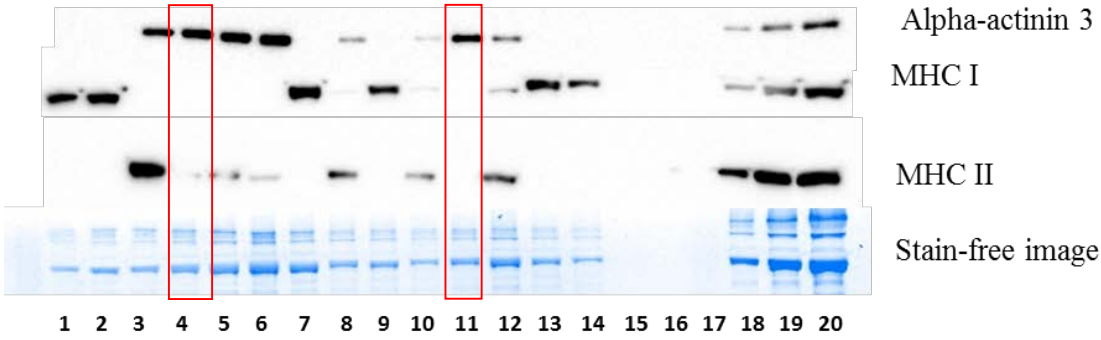
Figure 7.1 Representative blot of 14 single skeletal muscle fibres for fibre-typing. Each lane represents one fibre with its fibre-type classification based on either MHC I or MHCII abundance. Lanes which are outlined with a red box clearly show protein in a lane, yet neither the MHC I or II isoforms were detected.



The frequency of these fibres appearing on a gel ranged from sometimes not appearing up to 1-2 times per gel, but this was dependent on the participant. Given there was no other evidence or another approach to detect pure type IIx muscle fibre, these fibres were not included for analysis in this thesis. During the final two months of this thesis, western blots were conducted by the PhD candidate with the alpha-actinin3 antibody (rabbit, monoclonal, 1/10,000, Epitomics, 2316-1) for the detection of type IIx fibres, as it has previously been shown that alpha-actinin3 is almost exclusively expressed in type IIx fibres (North *et al.*, 1999; Norman *et al.*, 2009). There was some preliminary success in the detection of type IIx fibres with the use of the alpha-actinin3 antibody (Figure 7.2). However, the use of this antibody also has limitations as ~18-20% of the general population lack the alpha-actinin3 genotype ACTN3 (North *et al.*, 1999). Further, the alpha-actinin 3 antibody may not distinguish between IIa and IIx fibres (see Figure 7.2, lanes 3 and 8 for clear examples). Thus, the use of alpha-actinin3 for fibre-typing for type IIx fibres in single fibre western

blotting is only very preliminary and conclusions can't be made regarding its validity at this stage.

Figure 7.2 The same representative blot as Figure 7.1. Each lane represents one fibre with its fibre-type classification based on either MHC I or MHC II abundance. This image also shows the blot for the alpha-actinin 3 protein. In fibres with no blot for MHC I or II, show the alpha-actinin 3 protein.



Finally, following on from the confirmation of findings by Galpin *et al.*, (2012) regarding housekeeping protein GAPDH and the effects of the NKA isoforms in whole muscle when normalised to GAPDH (Chapter 4), future research should investigate other housekeeping proteins used to normalise proteins in human skeletal muscle research, such as beta-actin and beta-tubulin. This should include if any fibre-type specificity of these proteins exist and if or how they are affected by ageing. Further research into determining the best way to analyse and interpret western blotting data in an ageing sample compared to young is critical, given the reduction in GAPDH abundance and the observations that total protein may be reduced with ageing. This discovery was not made until late in this PhD candidacy, so a complete re-analysis and methodical overhaul was not conducted, but future research should determine whether there is a change in total protein and if so which is best way to normalise western blots in ageing research. Whether the total protein of every single fibre needs to be taken in to consideration before an analysis can be completed should be included in these investigations.

REFERENCES

Aagaard P, Suetta C, Caserotti P, Magnusson SP & Kjaer M. (2010). Role of the nervous system in sarcopenia and muscle atrophy with aging: strength training as a countermeasure. *Scandinavian journal of medicine & science in sports* **20,** 49-64.

Abdel-Azia MI, Manning W, Ward KM & Wareham AC. (1985). [3H]Ouabain binding in normal and dystrophic mouse skeletal muscles and the effect of age. *Journal of the Neurological Sciences* **70,** 47-53.

Adams GR, Hather BM & Dudley GA. (1994). Effect of short-term unweighting on human skeletal muscle strength and size. *Aviation, space, and environmental medicine* **65,** 1116-1121.

Allen DG, Lamb GD & Westerblad H. (2008). Skeletal Muscle Fatigue: Cellular Mechanisms. *Physiological reviews* **88,** 287-332.

Altenburg TM, Degens H, van Mechelen W, Sargeant AJ & de Haan A. (2007). *Recruitment of single muscle fibers during submaximal cycling exercise*, vol. 103.

Asano Y, Liberman U & Edelman I. (1976). Thyroid thermogenesis. Relationships between Na^+, K^+-dependent respiration and NaK-adenosine triphosphatase activity in rat skeletal muscle. *The Journal of Clinical Investigation* **57,** 368-379.

Aughey RJ. (2010). Australian Football Player Workrate: Evidence of fatigue and pacing? *International Journal of Sports Physiology & Performance* **5,** 394-405.

Aughey RJ, Clark SA, Gore CJ, Townsend NE, Hahn AG, Kinsman TA, Goodman C, Chow CM, Martin DT, Hawley JA & McKenna MJ. (2006). Interspersed normoxia during live high, train low interventions reverses an early reduction in muscle Na+, K+ATPase activity in well-trained athletes. *European Journal of Applied Physiology* **98,** 299-309.

Aughey RJ, Murphy KT, Clark SA, Garnham AP, Snow RJ, Cameron-Smith D, Hawley JA & McKenna MJ. (2007). Muscle Na+-K+-ATPase activity and isoform adaptations to intense interval exercise and training in well-trained athletes. *Journal of Applied Physiol* **103,** 39-47.

Ballak SB, Degens H, de Haan A & Jaspers RT. (2014). Aging related changes in determinants of muscle force generating capacity: A comparison of muscle aging in men and male rodents. *Ageing Research Reviews* **14,** 43-55.

Balog E, Thompson L & Fitts R. (1994). Role of sarcolemma action potentials and excitability in muscle fatigue. *Journal of Applied Physiology* **76,** 2157-2162.

Bangsbo J, Gunnarsson TP, Wendell J, Nybo L & Thomassen M. (2009). Reduced volume and increased training intensity elevate muscle Na+-K+ pump α2-subunit expression as well as short- and long-term work capacity in humans. *Journal of Applied Physiology* **107,** 1771-1780.

Bárány M. (1967). ATPase Activity of Myosin Correlated with Speed of Muscle Shortening. *The Journal of General Physiology* **50,** 197-218.

Barker AR, Day J, Smith A, Bond B & Williams CA. (2014). The influence of 2 weeks of low-volume high-intensity interval training on health outcomes in adolescent boys. *Journal of Sport Sciences* **32,** 757-765.

Benders AG, van-Kuppevelt TH, Oosterhof A, Wevers RA & Veerkamp JH. (1992). Adenosine triphosphatases during maturation of cultured human skeletal muscle cells and in adult human muscle. *Biochimica Et Biophysica Acta* **1112,** 89-98.

Benziane B, Widegren U, Pirkmajer S, Henriksson J, Stepto NK & Chibalin AV. (2011). Effect of exercise and training on phospholemman phosphorylation in human skeletal muscle. *American Journal of Physiology - Endocrinology And Metabolism*.

Berg HE, Dudley GA, Haggmark T, Ohlsen H & Tesch PA. (1991). Effects of lower limb unloading on skeletal muscle mass and function in humans. *Journal of Applied Physiology* **70,** 1882-1885.

Berg HE, Dudley GA, Hather B & Tesch PA. (1993). Work capacity and metabolic and morphologic characteristics of the human quadriceps muscle in response to unloading. *Clinical Physiology* **13,** 337-347.

Berg HE & Tesch PA. (1996). Changes in muscle function in response to 10 days of lower limb unloading in humans. *Acta physiologica Scandinavica* **157,** 63-70.

Bergstrom J, Guarnieri G & Hultman E. (1971). Carbohydrate metabolism and electrolyte changes in human muscle tissue during heavy work. *Journal of Applied Physiology* **30,** 122-125.

Bibert S, Roy S, Schaer D, Horisberger JD & Geering K. (2008). Phosphorylation of phospholemman (FXYD1) by protein kinases A and C modulates distinct Na,K-ATPase isozymes. *The Journal of biological chemistry* **283,** 476-486.

Blair SN, Kampert JB, Kohl HW, 3rd, Barlow CE, Macera CA, Paffenbarger RS, Jr. & Gibbons LW. (1996). Influences of cardiorespiratory fitness and other precursors on cardiovascular disease and all-cause mortality in men and women. *JAMA : the journal of the American Medical Association* **276,** 205-210.

Blanco G & Mercer RW. (1998). Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. *American Journal of Physiology - Renal Physiology* **275,** F633-F650.

Bogaev RC, Jia L, Kobayashi YM, Palmer CJ, Mounsey JP, Moorman JR, Jones LR & Tucker AL. (2001). Gene structure and expression of phospholemman in mouse. *Gene* **271,** 69-79.

Boon H, Kostovski E, Pirkmajer S, Song M, Lubarski I, Iversen PO, Hjeltnes N, Widegren U & Chibalin AV. (2012). Influence of chronic and acute spinal cord injury on skeletal muscle Na+-K+-ATPase and phospholemman expression in humans. *American journal of physiology Endocrinology and metabolism* **302,** E864-871.

Booth FW. (1977). Time course of muscular atrophy during immobilization of hindlimbs in rats. *Journal of applied physiology: respiratory, environmental and exercise physiology* **43,** 656-661.

Borg GA. (1982). Psychological bases of perceived exertion. *Medicine and science in sports and exercise* **14,** 377-381.

Bøttger P, Tracz Z, Heuck A, Nissen P, Romero-Ramos M & Lykke-Hartmann K. (2011). Distribution of Na/K-ATPase alpha 3 isoform, a sodium-potassium P-type pump associated with rapid-onset of dystonia parkinsonism (RDP) in the adult mouse brain. *The Journal of Comparative Neurology* **519,** 376-404.

Bottinelli R, Pellegrino MA, Canepari M, Rossi R & Reggiani C. (1999). Specific contributions of various muscle fibre types to human muscle performance: an in vitro study. *Journal of Electromyography and Kinesiology* **9,** 87-95.

Bray G, Wilcox W & Aguayo A. (1977). Ouabain binding sites in skeletal muscle from normal and dystrophic mice. *Journal of neurological sciences* **34,** 149-156.

Broman G, Quintana M, Lindberg T, Jansson E & Kaijser L. (2006). High intensity deep water training can improve aerobic power in elderly women. *European Journal of Applied Physiology* **98,** 117-123.

Bundgaard H, Schmidt TA, Larsen JS & Kjeldsen K. (1997). K+ supplementation increases muscle [Na+-K+-ATPase] and improves extrarenal K+homeostasis in rats. *Journal of Applied Physiology* **82,** 1136-1144.

Cairns SP, Hing WA, Slack JR, Mills RG & Loiselle DS. (1997). Different effects of raised [K+]o on membrane potential and contraction in mouse fast- and slow-twitch muscle. *The American journal of physiology* **273,** C598-611.

Cairns SP & Lindinger MI. (2008). Do multiple ionic interactions contribute to skeletal muscle fatigue? *The Journal of Physiology* **586,** 4039-4054.

Chin ER & Green HJ. (1993). Na^+-K^+ ATPase concentration in different adult rat skeletal muscles is related to oxidative potential. *Canadian journal of physiology and pharmacology* **71,** 315-318.

Clausen T. (2003a). Effects of age and exercise training on Na+-K+ pumps in skeletal muscle. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **285,** R720-R721.

Clausen T. (2003b). Na+-K+ pump regulation and skeletal muscle contractility. *Physiological reviews* **83,** 1269-1324.

Clausen T. (2010). Hormonal and pharmacological modification of plasma potassium homeostasis. *Fundamental & Clinical Pharmacology* **24,** 595-605.

Clausen T. (2013a). Excitation-induced exchange of Na+, K+, and Cl- in rat EDL muscle in vitro and in vivo: physiology and pathophysiology. *The Journal of General Physiology* **141,** 179-192.

Clausen T. (2013b). Quantification of Na+,K+ pumps and their transport rate in skeletal muscle: Functional significance. *Journal of General Physiology* **142,** 327-345.

Clausen T, Everts ME & Kjeldsen K. (1987). Quantification of the maximum capacity for active sodium-potassium transport in rat skeletal muscle. *The Journal of Physiology* **388,** 163-181.

Clausen T, Hansen O, Kjeldsen K & Nørgaard A. (1982). Effect of age, potassium depletion and denervation on specific displaceable [3H]ouabain binding in rat skeletal muscle in vivo. *The Journal of Physiology* **333,** 367-381.

Clausen T, Overgaard K & Nielsen OB. (2004). Evidence that the Na+-K+ leak/pump ratio contributes to the difference in endurance between fast- and slow-twitch muscles. *Acta physiologica Scandinavica* **180,** 209-216.

Coggan AR, Spina RJ, King DS, Rogers MA, Brown M, Nemeth PM & Holloszy JO. (1992). Skeletal muscle adaptations to endurance training in 60- to 70-yr-old men and women. *Journal of Applied Physiology* **72,** 1780-1786.

Cook SB, Brown KA, DeRuisseau K, Kanaley JA & Ploutz-Snyder LL. (2010). Skeletal muscle adaptations following blood flow-restricted training during 30 days of muscular unloading. *Journal of Applied Physiology* **109,** 341-349.

Cougnon M, Moseley A, Radzyukevich T, Lingrel J & Heiny J. (2002). Na,K-ATPase a- and ß-isoform expression in developing skeletal muscles: a₂ correlates with t-tubule formation. *Pflügers Archiv European Journal of Physiology* **445,** 123-131.

Crambert G, Fuzesi M, Garty H, Karlish S & Geering Kt. (2002). Phospholemman (FXYD1) associates with Na,K-ATPase and regulates its transport properties. *Proceedings of the National Academy of Sciences* **99,** 11476-11481.

Crambert G & Geering K. (2003). FXYD Proteins: New Tissue-Specific Regulators of the Ubiquitous Na,K-ATPase. *Sci STKE* **2003,** re1-.

Crambert G, Hasler U, Beggah AT, Yu C, Modyanov NN, Horisberger J-D, Lelièvre L & Geering K. (2000). Transport and Pharmacological Properties of Nine Different Human Na,K-ATPase Isozymes. *Journal of Biological Chemistry* **275,** 1976-1986.

Ditor DS, Hamilton S, Tarnopolsky MA, Green HJ, Craven BC, Parise G & Hicks AL. (2004). Na+,K+-ATPase concentration and fiber type distribution after spinal cord injury. *Muscle & nerve* **29,** 38-45.

Doganli C, Beck HC, Ribera AB, Oxvig C & Lykke-Hartmann K. (2013). α3Na+/K+-ATPase deficiency causes brain ventricle dilation and abrupt embryonic motility in zebrafish. *Journal of Biological Chemistry*.

Dorup I. (1996). Effects of K+, Mg2+ deficiency and adrenal steroids on Na+, K(+)-pump concentration in skeletal muscle. *Acta physiologica Scandinavica* **156,** 305-311.

Dørup I & Clausen T. (1997). Effects of adrenal steroids on the concentration of Na+–K+ pumps in rat skeletal muscle. *Journal of Endocrinology* **152,** 49-57.

Dørup I, Flyvbjerg A, Everts ME & Orskov H. (1992). Effects of growth hormone on growth and muscle Na(+)-K+ pump concentration in K(+)-deficient rats. *The American journal of physiology* **262,** E511-517.

Dulhunty A. (2006). Excitation-Contraction coupling from 1950s into the new millennium. *Clinical and Experimental Pharmacology and Physiology* **33,** 763-772.

Dutka TL & Lamb GD. (2007). Na+-K+ pumps in the transverse tubular system of skeletal muscle fibers preferentially use ATP from glycolysis. *American Journal of Physiology - Cell Physiology* **293,** C967-C977.

Dutka TL, Murphy RM, Stephenson DG & Lamb GD. (2008). Chloride conductance in the transverse tubular system of rat skeletal muscle fibres: importance in excitation contraction coupling and fatigue. *The Journal of Physiology* **586,** 875-887.

Edge J, Eynon N, McKenna MJ, Goodman CA, Harris RC & Bishop DJ. (2013). Altering the rest interval during high-intensity interval training does not affect muscle or performance adaptations. *Experimental physiology* **98,** 481-490.

Esfandiari S, Sasson Z & Goodman JM. (2014). Short-term high-intensity interval and continuous moderate-intensity training improve maximal aerobic power and diastolic filling during exercise. *European journal of applied physiology* **114,** 331-343.

Evans WJ. (2010). Skeletal muscle loss: cachexia, sarcopenia, and inactivity. *The American Journal of Clinical Nutrition* **91,** 1123S-1127S.

Everts ME & Clausen T. (1992). Activation of the Na-K pump by intracellular Na in rat slow- and fast-twitch muscle. *Acta physiologica Scandinavica* **145,** 353-362.

Everts ME & Clausen T. (1994). Excitation-induced activation of the Na(+)-K+ pump in rat skeletal muscle. *The American journal of physiology* **266,** C925-934.

Fleg JL & Lakatta EG. (1988). Role of muscle loss in the age-associated reduction in VO2 max. *Journal of Applied Physiology* **65,** 1147-1151.

Ford GA, Blaschke TF, Wiswell R & Hoffman BB. (1993). Effect of aging on changes in plasma potassium during exercise. *Journal of gerontology* **48,** 40-45.

Fowles JR, Green HJ & Ouyang J. (2004). Na+-K+-ATPase in rat skeletal muscle: content, isoform, and activity characteristics. *Journal of Applied Physiology* **96,** 316-326.

Frankenberg NT, Lamb GD, Vissing K & Murphy RM. (2013). Sub-cellular fractionation reveals HSP72 does not associate with SERCA in human skeletal muscle following damaging eccentric and concentric exercise. *Journal of Applied Physiology*.

Galuska D, Kotova O, Barrès R, Chibalina D, Benziane B & Chibalin AV. (2009). Altered expression and insulin-induced trafficking of Na+-K+-ATPase in rat skeletal muscle: effects of high-fat diet and exercise. *American Journal of Physiology - Endocrinology And Metabolism* **297,** E38-E49.

Geering K. (2006). FXYD proteins: new regulators of Na-K-ATPase. *American Journal of Physiology - Renal Physiology* **290,** F241-F250.

Geering K, BÉGuin P, Garty H, Karlish S, FÜZesi M, Horisberger J-D & Crambert G. (2003). FXYD Proteins: New Tissue- and Isoform-Specific Regulators of Na,K-ATPase. *Annals of the New York Academy of Sciences* **986,** 388-394.

Gibala MJ, Little JP, MacDonald MJ & Hawley JA. (2012). Physiological adaptations to low-volume, high-intensity interval training in health and disease. *The Journal of Physiology* **590,** 1077-1084.

Gibala MJ & McGee SL. (2008). Metabolic adaptations to short-term high-intensity interval training: a little pain for a lot of gain? *Exercise and sport sciences reviews* **36,** 58-63.

Gillen JB, Percival ME, Ludzki A, Tarnopolsky MA & Gibala MJ. (2013). Interval training in the fed or fasted state improves body composition and muscle oxidative capacity in overweight women. *Obesity (Silver Spring)*.

Green, Dahly, Shoemaker, Goreham, Bombardier & Ball B. (1999). Serial effects of high-resistance and prolonged endurance training on Na+–K+ pump concentration and enzymatic activities in human vastus lateralis. *Acta physiologica Scandinavica* **165,** 177-184.

Green HJ. (2004). Membrane excitability, weakness, and fatigue. *Canadian Journal of Applied Physiology* **29,** 291-307.

Green HJ, Chin ER, Ball-Burnett M & Ranney D. (1993). Increases in human skeletal muscle Na(+)-K(+)-ATPase concentration with short-term training. *American Journal of Physiology - Cell Physiology* **264,** C1538-C1541.

Green HJ, Duhamel TA, Stewart RD, Tupling AR & Ouyang J. (2008). Dissociation between changes in muscle Na+-K+-ATPase isoform abundance and activity with consecutive

days of exercise and recovery. *American Journal of Physiology - Endocrinology And Metabolism* **294,** E761-E767.

Green S, Langberg H, Skovgaard D, BÃ¼low J & KjÃ¦r M. (2000). Interstitial and arterialâ€“venous [K+] in human calf muscle during dynamic exercise: effect of ischaemia and relation to muscle pain. *The Journal of Physiology* **529,** 849-861.

Grounds MD, Radley HG, Lynch GS, Nagaraju K & De Luca A. (2008). Towards developing standard operating procedures for pre-clinical testing in the mdx mouse model of Duchenne muscular dystrophy. *Neurobiology of Disease* **31,** 1-19.

Gullestad L, Hallén J & Sejersted OM. (1995). K^+ balance of the quadriceps muscle during dynamic exercise with and without beta-adrenoceptor blockade. *Journal of Applied Physiology* **78,** 513-523.

Gunnarsson TP & Bangsbo J. (2012). The 10-20-30 training concept improves performance and health profile in moderately trained runners. *Journal of Applied Physiology*.

Hackney KJ & Ploutz-Snyder LL. (2012). Unilateral lower limb suspension: integrative physiological knowledge from the past 20 years (1991-2011). *European journal of applied physiology* **112,** 9-22.

Hallén J. (1996). K+ balance in humans during exercise. *Acta physiologica Scandinavica* **156,** 279-286.

Haller RG, Clausen T & Vissing J. (1998). Reduced levels of skeletal muscle Na^+K^+ -ATPase in McArdle disease. *Neurology* **50,** 37-40.

Hansen O. (1984). Interaction of cardiac glycosides with (Na+ + K+)-activated ATPase. A biochemical link to digitalis-induced inotropy. *Pharmacological Reviews* **36,** 143-163.

Hansen O. (2001). The α1 isoform of Na+,K+-ATPase in rat soleus and extensor digitorum longus. *Acta physiologica Scandinavica* **173,** 335-341.

Hansen O & Clausen T. (1988). Quantitative determination of Na+-K+-ATPase and other sarcolemmal components in muscle cells. *American Journal of Physiology - Cell Physiology* **254,** C1-C7.

Harber MP, Konopka AR, Douglass MD, Minchev K, Kaminsky LA, Trappe TA & Trappe S. (2009). Aerobic exercise training improves whole muscle and single myofiber size

and function in older women. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **297,** R1452-R1459.

Harber MP, Konopka AR, Undem MK, Hinkley JM, Minchev K, Kaminsky LA, Trappe TA & Trappe S. (2012). Aerobic exercise training induces skeletal muscle hypertrophy and age-dependent adaptations in myofiber function in young and older men. *Journal of Applied Physiology* **113,** 1495-1504.

Harmer AR, Ruell PA, McKenna MJ, Chisholm DJ, Hunter SK, Thom JM, Morris NR & Flack JR. (2006). Effects of sprint training on extrarenal potassium regulation with intense exercise in Type 1 diabetes. *Journal of Applied Physiology* **100,** 26-34.

Harrison AP, Clausen T, Duchamp C & Dauncey MJ. (1994). Roles of skeletal muscle morphology and activity in determining Na(+)-K(+)-ATPase concentration in young pigs. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **266,** R102-R111.

He S, Shelly DA, Moseley AE, James PF, James JH, Paul RJ & Lingrel JB. (2001). The α1- and α2-isoforms of Na-K-ATPase play different roles in skeletal muscle contractility. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **281,** R917-R925.

Helge EW, Aagaard P, Jakobsen MD, Sundstrup E, Randers MB, Karlsson MK & Krustrup P. (2010). Recreational football training decreases risk factors for bone fractures in untrained premenopausal women. *Scandinavian Journal of Medicine & Science in Sports* **20,** 31-39.

Hodgkin AL & Horowicz P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *The Journal of Physiology* **148,** 127-160.

Hundal HS, Marette A, Mitsumoto Y, Ramlal T, Blostein R & Klip A. (1992). Insulin induces translocation of the alpha 2 and beta 1 subunits of the Na+/K(+)-ATPase from intracellular compartments to the plasma membrane in mammalian skeletal muscle. *Journal of Biological Chemistry* **267,** 5040-5043.

Hundal HS, Marette A, Ramlal T, Liu Z & Klip A. (1993). Expression of β subunit isoforms of the Na+,K+-ATPase is muscle type-specific. *FEBS Letters* **328,** 253-258.

Hundal HS, Maxwell DL, Ahmed A, Darakhshan F, Mitsumoto Y & Klip A. (1994). Subcellular distribution and immunocytochemical localization of Na,K-ATPase subunit isoforms in human skeletal muscle. *Molecular membrane biology* **11,** 255-262.

Hurley BF, Hanson ED & Sheaff AK. (2011). Strength Training as a Countermeasure to Aging Muscle and Chronic Disease. *Sports Medicine* **41,** 289-306.

Iaia FM & Bangsbo J. (2010). Speed endurance training is a powerful stimulus for physiological adaptations and performance improvements of athletes. *Scandinavian Journal of Medicine & Science in Sports* **20,** 11-23.

Iaia FM, Perez-Gomez J, Thomassen M, Nordsborg NB, Hellsten Y & Bangsbo J. (2011). Relationship between performance at different exercise intensities and skeletal muscle characteristics. *Journal of Applied Physiology* **110,** 1555-1563.

Iaia FM, Thomassen M, Kolding H, Gunnarsson T, Wendell J, Rostgaard T, Nordsborg N, Krustrup P, Nybo L, Hellsten Y & Bangsbo J. (2008). Reduced volume but increased training intensity elevates muscle Na+-K+ pump α1-subunit and NHE1 expression as well as short-term work capacity in humans. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **294,** R966-R974.

Ingwersen M, Kristensen M, Pilegaard H, Wojtaszewski J, Richter E & Juel C. (2011). Na,K-ATPase Activity in Mouse Muscle is Regulated by AMPK and PGC-1α. *Journal of Membrane Biology* **242,** 1-10.

Jebens E, Steen H, Fjeld TO, Bye E & Sejersted OM. (1995). Changes in Na+, K(+)-adenosinetriphosphatase, citrate synthase and K+ in sheep skeletal muscle during immobilization and remobilization. *European journal of applied physiology and occupational physiology* **71,** 386-395.

Jennings D, Cormack SJ, Coutts AJ & Aughey RJ. (2012). GPS analysis of an international field hockey tournament. *International journal of sports physiology and performance* **7,** 224-231.

Johnson ML, Robinson MM & Nair KS. (2013). Skeletal muscle aging and the mitochondrion. *Trends in Endocrinology & Metabolism* **24,** 247-256.

Juel C, Pilegaard H, Nielsen JJ & Bangsbo J. (2000). Interstitial K+ in human skeletal muscle during and after dynamic graded exercise determined by microdialysis. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **278,** R400-R406.

Kimm SY, Glynn NW, McMahon RP, Voorhees CC, Striegel-Moore RH & Daniels SR. (2006). Self-perceived barriers to activity participation among sedentary adolescent girls. *Medicine and science in sports and exercise* **38,** 534-540.

Kjeldsen K, Everts ME & Clausen T. (1986). The effects of thyroid hormones on 3H-ouabain binding site concentration, Na,K-contents and 86Rb-efflux in rat skeletal muscle. *Pflugers Archiv : European journal of physiology* **406,** 529-535.

Kjeldsen K, Nørgaard A & Clausen T. (1984a). The age-dependent changes in the number of 3H-ouabain binding sites in mammalian skeletal muscle. *Pflugers Archiv European Journal of Physiology* **402,** 100-108.

Kjeldsen K, NØRgaard A & Clausen T. (1984b). Effect of K-depletion on 3H-ouabain binding and Na–K-contents in mammalian skeletal muscle. *Acta Physiologica Scandinavica* **122,** 103-117.

Kjeldsen K, Norgaard A, Gotzsche CO, Thomassen A & Clausen T. (1984c). Effect of thyroid function on number of Na-K pumps in human skeletal muscle. *Lancet* **2,** 8-10.

Klitgaard H & Clausen T. (1989). Increased total concentration of Na-K pumps in vastus lateralis muscle of old trained human subjects. *Journal of Applied Physiology* **67,** 2491-2494.

Kohrt WM, Malley MT, Coggan AR, Spina RJ, Ogawa T, Ehsani AA, Bourey RE, Martin WH & Holloszy JO. (1991). Effects of gender, age, and fitness level on response of VO2max to training in 60-71 yr olds. *Journal of Applied Physiology* **71,** 2004-2011.

Konopka AR, Douglass MD, Kaminsky LA, Jemiolo B, Trappe TA, Trappe S & Harber MP. (2010). Molecular Adaptations to Aerobic Exercise Training in Skeletal Muscle of Older Women. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* **65A,** 1201-1207.

Kristensen M & Juel C. (2009). Potassium-transporting proteins in skeletal muscle: cellular location and fibre-type differences. *Acta Physiologica* **198,** 105-123.

Kristensen M & Juel C. (2010). Na+,K+-ATPase Na+ Affinity in Rat Skeletal Muscle Fiber Types. *Journal of Membrane Biology* **234,** 35-45.

Krustrup P, Dvorak J, Junge A & Bangsbo J. (2010). Executive summary: The health and fitness benefits of regular participation in small-sided football games. *Scandinavian Journal of Medicine & Science in Sports* **20,** 132-135.

Krustrup P, Randers MB, Andersen LJ, Jackman SR, Bangsbo J & Hansen PR. (2013). Soccer improves fitness and attenuates cardiovascular risk factors in hypertensive men. *Medicine and science in sports and exercise* **45,** 553-560.

Kwak HB. (2013). Aging, exercise, and extracellular matrix in the heart. *Journal of exercise rehabilitation* **9,** 338-347.

Lamb G. (2009). Mechanisms of excitation-contraction uncoupling relevant to activity-induced muscle fatigue. *Appl Physio Nutr Metab* **34,** 368-372.

Lamboley CR, Murphy RM, McKenna MJ & Lamb GD. (2013). Endogenous and maximal sarcoplasmic reticulum calcium content and calsequestrin expression in type I and type II human skeletal muscle fibres. *The Journal of Physiology* **591,** 6053-6068.

Lavoie L, He L, Ramlal T, Ackerley C, Marette A & Klip A. (1995). The GLUT4 glucose transporter and the α2 subunit of the Na+,K+-ATPase do not localize to the same intracellular vesicles in rat skeletal muscle. *FEBS Letters* **366,** 109-114.

Lavoie L, Levenson R, Martin-Vasallo P & Klip A. (1997). The Molar Ratios of α and β Subunits of the Na+−K+-ATPase Differ in Distinct Subcellular Membranes from Rat Skeletal Muscle†. *Biochemistry* **36,** 7726-7732.

Lavoie L, Roy D, Ramlal T, Dombrowski L, Martn-Vasallo P, Marette A, Carpentier JL & Klip A. (1996). Insulin-induced translocation of Na(+)-K(+)-ATPase subunits to the plasma membrane is muscle fiber type specific. *American Journal of Physiology - Cell Physiology* **270,** C1421-C1429.

Leenders M, Verdijk LB, van der Hoeven L, van Kranenburg J, Nilwik R & van Loon LJC. (2013). Elderly Men and Women Benefit Equally From Prolonged Resistance-Type Exercise Training. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* **68,** 769-779.

Leivseth G, Clausen T, Everts ME & Bjordal E. (1992). Effects of reduced joint mobility and training on Na,K-ATPase and Ca-ATPase in skeletal muscle. *Muscle & nerve* **15,** 843-849.

Leivseth G & Reikeras O. (1994). Changes in muscle fiber cross-sectional area and concentrations of Na,K-ATPase in deltoid muscle in patients with impingement syndrome of the shoulder. *The Journal of orthopaedic and sports physical therapy* **19,** 146-149.

Lepley LK & Palmieri-Smith R. (2013). Effect of eccentric strengthening after anterior cruciate ligament reconstruction on quadriceps strength. *Journal of sport rehabilitation* **22,** 150-156.

Lexell J, Taylor C & M. S. (1988). What is the cause of the ageing atrophy? Total number, size and proportion of different fiber types studied in whole vastus lateralis muscle from 15- to 83-year-old men. *J Neurol Sci* **84,** 275-294.

Lindinger MI. (1995). Potassium regulation during exercise and recovery in humans: Implications for skeletal and cardiac muscle. *Journal of Molecular and Cellular Cardiology* **27,** 1011-1022.

Lingrel J, Moseley AMY, Dostanic IVA, Cougnon M, He S, James P, Woo A, O'Connor K & Neumann J. (2003). Functional Roles of the α Isoforms of the Na,K-ATPase. *Annals of the New York Academy of Sciences* **986,** 354-359.

Lingrel JB, Williams MT, Vorhees CV & Moseley AE. (2007). Na,K-ATPase and the role of alpha isoforms in behavior. *Journal of bioenergetics and biomembranes* **39,** 385-389.

Lowe DA, Degens H, Chen KD & Alway SE. (2000). Glyceraldehyde-3-Phosphate Dehydrogenase Varies With Age in Glycolytic Muscles of Rats. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* **55,** B160-B164.

Lunt H, Draper N, Marshall HC, Logan FJ, Hamlin MJ, Shearman JP, Cotter JD, Kimber NE, Blackwell G & Frampton CM. (2014). High intensity interval training in a real world setting: a randomized controlled feasibility study in overweight inactive adults, measuring change in maximal oxygen uptake. *PLoS One* **9,** e83256.

Masuda S, Hayashi T, Egawa T & Taguchi S. (2009). Evidence for differential regulation of lactate metabolic properties in aged and unloaded rat skeletal muscle. *Experimental Gerontology* **44,** 280-288.

McDonough AA, Thompson CB & Youn JH. (2002). Skeletal muscle regulates extracellular potassium. *American Journal of Physiology - Renal Physiology* **282,** F967-F974.

McKenna MJ, Bangsbo J & Renaud J-M. (2008). Muscle K+, Na+, and Cl disturbances and Na+-K+ pump inactivation: implications for fatigue. *Journal of Applied Physiology* **104,** 288-295.

McKenna MJ, Heigenhauser GJ, McKelvie RS, MacDougall JD & Jones NL. (1997). Sprint training enhances ionic regulation during intense exercise in men. *The Journal of Physiology* **501,** 687-702.

McKenna MJ, Medved I, Goodman CA, Brown MJ, Bjorksten AR, Murphy KT, Petersen AC, Sostaric S & Gong X. (2006). N-acetylcysteine attenuates the decline in muscle

Na+,K+-pump activity and delays fatigue during prolonged exercise in humans. *The Journal of Physiology* **576,** 279-288.

McKenna MJ, Perry BD, Serpiello FR, Caldow MK, Levinger P, Cameron-Smith D & Levinger I. (2012). Unchanged [3H]ouabain binding site content but reduced Na+-K+ pump α2-protein abundance in skeletal muscle in older adults. *Journal of Applied Physiology* **113,** 1505-1511.

McKenna MJ, Schmidt TA, Hargreaves M, Cameron L, Skinner SL & Kjeldsen K. (1993). Sprint training increases human skeletal muscle Na(+)-K(+)-ATPase concentration and improves K+ regulation. *Journal of Applied Physiology* **75,** 173-180.

Medbo JI, Jebens E, Vikne H, Refsnes PE & Gramvik P. (2001). Effect of strenuous strength training on the Na-K pump concentration in skeletal muscle of well-trained men. *Eur J Appl Physiol* **84,** 148-154.

Meredith CN, Frontera WR, Fisher EC, Hughes VA, Herland JC, Edwards J & Evans WJ. (1989). Peripheral effects of endurance training in young and old subjects. *Journal of Applied Physiology* **66,** 2844-2849.

Mishra SK & Misra V. (2003). Muscle sarcopenia: an overview. *Acta myologica : myopathies and cardiomyopathies : official journal of the Mediterranean Society of Myology / edited by the Gaetano Conte Academy for the study of striated muscle diseases* **22,** 43-47.

Mohr M, Krustrup P, Nielsen JJ, Nybo L, Rasmussen MK, Juel C & Bangsbo J. (2007). Effect of two different intense training regimens on skeletal muscle ion transport proteins and fatigue development. *American journal of physiology Regulatory, integrative and comparative physiology* **292,** R1594-1602.

Mollica JP, Oakhill JS, Lamb GD & Murphy RM. (2009). Are genuine changes in protein expression being overlooked? Reassessing Western blotting. *Analytical Biochemistry* **386,** 270-275.

Murphy KT, Aughey RJ, Petersen AC, Clark SA, Goodman C, Hawley JA, Cameron-Smith D, Snow RJ & McKenna MJ. (2007). Effects of endurance training status and sex differences on Na+,K+-pump mRNA expression, content and maximal activity in human skeletal muscle. *Acta Physiologica* **189,** 259-269.

Murphy KT, Petersen AC, Goodman C, Gong X, Leppik JA, Garnham AP, Cameron-Smith D, Snow RJ & McKenna MJ. (2006a). Prolonged submaximal exercise induces isoform-specific Na+-K+-ATPase mRNA and protein responses in human skeletal

muscle. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **290,** R414-R424.

Murphy KT, Snow RJ, Petersen AC, Murphy RM, Mollica J, Lee JS, Garnham AP, Aughey RJ, Leppik JA, Medved I, Cameron-Smith D & McKenna MJ. (2004). Intense exercise up-regulates Na+,K+-ATPase isoform mRNA, but not protein expression in human skeletal muscle. *The Journal of Physiology* **556,** 507-519.

Murphy RM. (2011). Enhanced technique to measure proteins in single segments of human skeletal muscle fibers: fiber-type dependence of AMPK-alpha1 and -beta1. *Journal of Applied Physiology* **110,** 820-825.

Murphy RM & Lamb GD. (2013). Important considerations for protein analyses using antibody based techniques: Down-sizing western blotting up-sizes outcomes. *The Journal of Physiology*.

Murphy RM, Mollica JP, Beard NA, Knollmann BC & Lamb GD. (2011). Quantification of calsequestrin 2 (CSQ2) in sheep cardiac muscle and Ca2+-binding protein changes in CSQ2 knockout mice. *American Journal of Physiology - Heart and Circulatory Physiology* **300,** H595-H604.

Murphy RM, Verburg E & Lamb GD. (2006b). Ca2+ activation of diffusible and bound pools of μ-calpain in rat skeletal muscle. *The Journal of Physiology* **576,** 595-612.

Nelson M, Rejeski J, Balir S, Duncan P, Judge J, King A, Macera C & Castaneda-Sceppa C. (2007). Physical Activity and Public Health in Older Adults: Recommendation from the American College of Sports Medicine and the American Heart Association. *Medicine & Science in Sports & Exercise* **39,** 1435-1445.

Nemoto K-i, Gen-no H, Masuki S, Okazaki K & Nose H. (2007). Effects of High-Intensity Interval Walking Training on Physical Fitness and Blood Pressure in Middle-Aged and Older People. *Mayo Clinic Proceedings* **82,** 803-811.

Ng Y-C, Nagarajan M, Jew KN, Mace LC & Moore RL. (2003). Exercise training differentially modifies age-associated alteration in expression of Na+-K+-ATPase subunit isoforms in rat skeletal muscles. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **285,** R733-R740.

Nielsen B & Paoli F. (2007). Regulation of Na^+ -K^+ homeostatsis and excitabilty in contracting muscles: implications for fatigue. *Appl Physio Nutr Metab* **32,** 974-984.

Nielsen JJ, Mohr M, Klarskov C, Kristensen M, Krustrup P, Juel C & Bangsbo J. (2004). Effects of high-intensity intermittent training on potassium kinetics and performance in human skeletal muscle. *The Journal of Physiology* **554,** 857-870.

Nielsen O & Clausen T. (2000). The Na^+/K^+-Pump protects muscle excitability and contractility during exercise *Exercise and Sport Sciences Reviews* **28,** 159-164.

Nordsborg N, Goodmann C, McKenna MJ & Bangsbo J. (2005a). Dexamethasone up-regulates skeletal muscle maximal Na+,K+ pump activity by muscle group specific mechanisms in humans. *The Journal of Physiology* **567,** 583-589.

Nordsborg N, Thomassen M, Lundby C, Pilegaard H & Bangsbo J. (2005b). Contraction-induced increases in Na+-K+-ATPase mRNA levels in human skeletal muscle are not amplified by activation of additional muscle mass. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **289,** R84-R91.

Nørgaard A, Kjeldsen K & Clausen T. (1984). A method for determination of the total number of [^{3}H]-ouabain binding sites in biopsies of human skeletal muscle. *Scandinavian journal of clinical and laboratory investigation* **44,** 509-518.

Norman B, Esbjörnsson M, Rundqvist H, Österlund T, von Walden F & Tesch PA. (2009). Strength, power, fiber types, and mRNA expression in trained men and women with different ACTN3 R577X genotypes. *Journal of Applied Physiology* **106,** 959-965.

North KN, Yang N, Wattanasirichaigoon D, Mills M, Easteal S & Beggs AH. (1999). A common nonsense mutation results in alpha-actinin-3 deficiency in the general population. *Nature genetics* **21,** 353-354.

Ogawa T, Spina RJ, Martin WH, Kohrt WM, Schechtman KB, Holloszy JO & Ehsani AA. (1992). Effects of aging, sex, and physical training on cardiovascular responses to exercise. *Circulation* **86,** 494-503.

Payne AM & Delbono O. (2004). Neurogenesis of Excitation-Contraction uncoupling in aging skeletal muscle. *Exercise & Sport Sciences Reviews* **32,** 36-40.

Pedersen TH, de Paoli F & Nielsen OB. (2005). Increased Excitability of Acidified Skeletal Muscle. *The Journal of General Physiology* **125,** 237-246.

Perry BD, Levinger P, Serpiello FR, Caldow MK, Cameron-Smith D, Bartlett JR, Feller JA, Bergman NR, Levinger I & McKenna MJ. (2013). The effects of osteoarthritis and age on skeletal muscle strength, Na+-K+-ATPase content, gene and isoform expression. *Journal of Applied Physiology* **115,** 1443-1449.

Petersen AC, Leikis MJ, McMahon LP, Kent AB, Murphy KT, Gong X & McKenna MJ. (2012). Impaired exercise performance and muscle Na+,K+-pump activity in renal transplantation and haemodialysis patients. *Nephrology Dialysis Transplantation* **27,** 2036-2043.

Porter MM, Vandervoort AA & Lexell J. (1995). Aging of human muscle: structure, function and adaptability. *Scandinavian Journal of Medicine & Science in Sports* **5,** 129-142.

Proctor DN & Joyner MJ. (1997). Skeletal muscle mass and the reduction ofV˙o 2 max in trained older subjects. *Journal of Applied Physiology* **82,** 1411-1415.

Radzyukevich TL, Neumann JC, Rindler TN, Oshiro N, Goldhamer DJ, Lingrel JB & Heiny JA. (2013). Tissue-specific role of the Na,K-ATPase alpha2 isozyme in skeletal muscle. *The Journal of biological chemistry* **288,** 1226-1237.

Rampinini E, Coutts A, Castagna C, Sassi R & Impellizzeri M. (2007). Variation in top level soccer match performance *International Journal of Sports Medicine* **28,** 1018-1024.

Rasmussen MK, Juel C & Nordsborg NB. (2011). Exercise-induced regulation of muscular Na+-K+ pump, FXYD1, and NHE1 mRNA and protein expression: importance of training status, intensity, and muscle type. *American journal of physiology Regulatory, integrative and comparative physiology* **300,** R1209-1220.

Rasmussen MK, Kristensen M & Juel C. (2008). Exercise-induced regulation of phospholemman (FXYD1) in rat skeletal muscle: implications for Na+/K+-ATPase activity. *Acta Physiologica* **194,** 67-79.

Reis J, Zhang L, Cala S, Jew KN, Mace LC, Chung L, Moore RL & Ng Y-C. (2005). Expression of phospholemman and its association with Na+-K+-ATPase in skeletal muscle: effects of aging and exercise training. *Journal of Applied Physiology* **99,** 1508-1515.

Rich MM & Pinter MJ. (2003). Crucial Role of Sodium Channel Fast Inactivation in Muscle Fibre Inexcitability in a Rat Model of Critical Illness Myopathy. *The Journal of Physiology* **547,** 555-566.

Röckl KSC, Witczak CA & Goodyear LJ. (2008). Signaling mechanisms in skeletal muscle: Acute responses and chronic adaptations to exercise. *IUBMB Life* **60,** 145-153.

Rognmo O, Hetland E, Hetland J, Hoff J & Slordahl S. (2004). High intensity aerobic interval training exercise is superior to moderate intensity exercise for increasing aerobic

capacity in patients in patients with coronary artery disease. *European society of cardiovascular prevention and rehabiliation* **11,** 216-222.

Schmidt TA, Hasselbalch S, Farrell PA, Vestergaard H & Kjeldsen K. (1994). Human and rodent muscle Na^+-K^+-ATPase in diabetes related to insulin, starvation, and training. *J Appl Physiol* **76,** 2140-2146.

Schulze K, Gallagher P & Trappe S. (2002). Resistance training preserves skeletal muscle function during unloading in humans. *Medicine and science in sports and exercise* **34,** 303-313.

Seals DR, Hagberg JM, Hurley BF, Ehsani AA & Holloszy JO. (1984). Endurance training in older men and women. I. Cardiovascular responses to exercise. *Journal of Applied Physiology* **57,** 1024-1029.

Sejersted OM & Sjøgaard G. (2000). Dynamics and Consequences of Potassium Shifts in Skeletal Muscle and Heart During Exercise. *Physiological reviews* **80,** 1411-1481.

Serpiello F, McKenna M, Stepto N, Bishop D & Aughey R. (2011). Performance and physiological responses to repeated-sprint exercise: a novel multiple-set approach. *Eur J Appl Physiol* **111,** 669-678.

Serpiello FR, McKenna MJ, Bishop DJ, Aughey RJ, Caldow MK, Cameron-Smith D & Stepto NK. (2012). Repeated sprints alter signaling related to mitochondrial biogenesis in humans. *Medicine and science in sports and exercise* **44,** 827-834.

Sjøgaard G. (1983). Electrolytes in slow and fast muscle fibers of humans at rest and with dynamic exercise. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **245,** R25-R31.

Sjøgaard G. (1986). Water and electrolyte fluxes during exercise and their relation to muscle fatigue. *Acta physiologica Scandinavica Supplementum* **556,** 129-136.

Sjøgaard G. (1996). Potassium and fatigue: the pros and cons. *Acta physiologica Scandinavica* **156,** 257-264.

Sjøgaard G, Adams RP & Saltin B. (1985). Water and ion shifts in skeletal muscle of humans with intense dynamic knee extension. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **248,** R190-R196.

Skelton DA, Greig CA, Davies JM & Young A. (1994). Strength, power and related functional ability of healthy people aged 65-89 years. *Age Ageing* **23,** 371-377.

Smart N. (2013). How do cardiorespiratory fitness improvements vary with physical training modality in heart failure patients? A quantitative guide. *Experimental and clinical cardiology* **18,** 21-25.

Spencer M, Bishop D, Dawson B & Goodman C. (2005). Physiological and metabolic responses of repeated-sprint activities:specific to field-based team sports. *Sports medicine* **35,** 1025-1044.

Stutts WC. (2002). Physical activity determinants in adults. Perceived benefits, barriers, and self efficacy. *AAOHN journal : official journal of the American Association of Occupational Health Nurses* **50,** 499-507.

Sun X, Nagarajan M, Beesley PW & Ng Y-C. (1999). Age-associated differential expression of Na+-K+-ATPase subunit isoforms in skeletal muscles of F-344/BN rats. *Journal of Applied Physiology* **87,** 1132-1140.

Suominen H, Heikkinen E & Parkatti T. (1977). Effect of eight weeks' physical training on muscle and connective tissue of the M. vastus lateralis in 69-year-old men and women. *Journal of gerontology* **32,** 33-37.

Tesch PA, Trieschmann JT & Ekberg A. (2004). Hypertrophy of chronically unloaded muscle subjected to resistance exercise. *Journal of Applied Physiology* **96,** 1451-1458.

Therien AG & Blostein R. (2000). Mechanisms of sodium pump regulation. *American Journal of Physiology - Cell Physiology* **279,** C541-C566.

Thomassen M, Christensen PM, Gunnarsson TP, Nybo L & Bangsbo J. (2010). Effect of 2-wk intensified training and inactivity on muscle Na+-K+ pump expression, phospholemman (FXYD1) phosphorylation, and performance in soccer players. *Journal of Applied Physiology* **108,** 898-905.

Thomassen M, Murphy RM & Bangsbo J. (2013). Fibre type specific change in FXYD1 phosphorylation during acute intense exercise in humans. *The Journal of Physiology*.

Thomassen M, Rose AJ, Jensen TE, Maarbjerg SJ, Bune L, Leitges M, Richter EA, Bangsbo J & Nordsborg NB. (2011). Protein kinase Cα activity is important for contraction-induced FXYD1 phosphorylation in skeletal muscle. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **301,** R1808-R1814.

Thompson CB, Dorup I, Ahn J, Leong PKK & McDonough AA. (2001). Glucocorticoids increase sodium pump α2- and β1-subunit abundance and mRNA in rat skeletal muscle. *American Journal of Physiology - Cell Physiology* **280,** C509-C516.

Thompson CB & McDonough AA. (1996a). Skeletal Muscle Na,K-ATPase alpha and beta Subunit Protein Levels Respond to Hypokalemic Challenge with Isoform and Muscle Type Specificity. *Journal of Biological Chemistry* **271,** 32653-32658.

Thompson CB & McDonough AA. (1996b). Skeletal muscle Na,K-ATPase alpha and beta subunit protein levels respond to hypokalemic challenge with isoform and muscle type specificity. *The Journal of biological chemistry* **271,** 32653-32658.

Tjønna AE, Leinan IM, Bartnes AT, Jenssen BM, Gibala MJ, Winett RA & Wisløff U. (2013). Low- and High-Volume of Intensive Endurance Training Significantly Improves Maximal Oxygen Uptake after 10-Weeks of Training in Healthy Men. *PLoS ONE* **8,** e65382.

Tomczak CR, Thompson RB, Paterson I, Schulte F, Cheng-Baron J, Haennel RG & Haykowsky MJ. (2011). Effect of acute high-intensity interval exercise on postexercise biventricular function in mild heart failure. *Journal of Applied Physiology* **110,** 398-406.

Trapp EG, Chisholm DJ, Freund J & Boutcher SH. (2008). The effects of high-intensity intermittent exercise training on fat loss and fasting insulin levels of young women. *International journal of obesity* **32,** 684-691.

Trappe SW, Trappe TA, Lee GA & Costill DL. (2001). Calf muscle strength in humans. *International journal of sports medicine* **22,** 186-191.

Varley MC, Gabbett T & Aughey RJ. (2013). Activity profiles of professional soccer, rugby league and Australian football match play. *Journal of Sports Sciences***,** 1-9.

Wang J, Velotta JB, McDonough AA & Farley RA. (2001). All human Na+-K+-ATPase α-subunit isoforms have a similar affinity for cardiac glycosides. *American Journal of Physiology - Cell Physiology* **281,** C1336-C1343.

Welle S, Thornton C & Statt M. (1995). Myofibrillar protein synthesis in young and old human subjects after three months of resistance training. *The American journal of physiology* **268,** E422-427.

Wells GD, Selvadurai H & Tein I. (2009). Bioenergetic provision of energy for muscular activity. *Paediatric Respiratory Reviews* **10,** 83-90.

Westerblad H, Bruton JD & Katz A. (2010). Skeletal muscle: Energy metabolism, fiber types, fatigue and adaptability. *Experimental Cell Research* **316,** 3093-3099.

Weston KS, Wisloff U & Coombes JS. (2013). High-intensity interval training in patients with lifestyle-induced cardiometabolic disease: a systematic review and meta-analysis. *British journal of sports medicine*.

Widrick JJ, Trappe SW, Romatowski JG, Riley DA, Costill DL & Fitts RH. (2002). Unilateral lower limb suspension does not mimic bed rest or spaceflight effects on human muscle fiber function. *Journal of Applied Physiology* **93,** 354-360.

Wisloff U, Stoylen A, Loennechen JP, Bruvold M, Rognmo O, Haram PM, Tjonna AE, Helgerud J, Slordahl SA, Lee SJ, Videm V, Bye A, Smith GL, Najjar SM, Ellingsen O & Skjaerpe T. (2007). Superior Cardiovascular Effect of Aerobic Interval Training Versus Moderate Continuous Training in Heart Failure Patients: A Randomized Study. *Circulation* **115,** 3086-3094.

Zhang L, Morris KJ & Ng Y-C. (2006). Fiber type-specific immunostaining of the Na+,K+-ATPase subunit isoforms in skeletal muscle: Age-associated differential changes. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1762,** 783-793.