Exercise Training, Immobilisation and Castration Effects on Skeletal Muscle Na^+,K^+-ATPase

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Submitted by

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Abstract

The Na^+,K^+-ATPase (NKA) is a key protein involved in the maintenance of skeletal muscle excitability during contractions, and comprises two subunits (α and β), each of which are expressed as multiple isoforms in skeletal muscle (α_1 - α_3 and β_1 - β_3). Therefore any modulation of NKA content or of individual NKA isoforms has the potential to affect muscle function. This thesis comprises four studies that investigated the effects of conditions intended to induce downregulation or upregulation of the NKA in skeletal muscle, utilising hindlimb immobilisation, testosterone suppression and increased physical activity through various training regimes. A theme of the thesis was specific effects of changes in physical activity and on muscle NKA.

Study one. This study investigated the effects of hindlimb immobilisation and testosterone suppression via castration surgery on soleus muscle [^{3}H]ouabain binding site content and NKA isoform abundances. Eight week old male Fischer rats underwent sham or castration surgery, and then after 7 days were subjected to 10 days of immobilisation of one hindlimb. For both sham and castration groups, soleus muscles were obtained 7 days after surgery from non-immobilised controls, following 10 days immobilisation and after 14 days of recovery, from both the cast and non-cast leg. Within the sham group, after immobilisation, the [^{3}H]ouabain binding site content in the cast leg was 26% lower than in the non-cast leg ($p = 0.023$) and 34% lower ($p = 0.001$) than in the non-immobilised control group ($P = 0.012$), but did not differ at 14 days recovery compared to either the non-cast leg or non-immobilised control group. There were no differences in the NKA α_1, α_2, α_3, β_1 or β_2 isoform abundances in the cast leg compared to either the non-cast leg, or the non-immobilised control group, after immobilisation, or at 14 days recovery. Within the castration group, the [^{3}H]ouabain binding site content in the cast leg after immobilisation was 34% lower ($p= 0.001$) than in the non-immobilised control

group and remained depressed by 34% ($p = 0.001$) at 14 days recovery after immobilisation. The α_2 isoform in the cast leg was 60% lower than in both the non-cast leg ($p = 0.004$) and non-immobilised control group ($p = 0.004$) and remained 42% lower than the non-immobilised control group at 14 days recovery ($p = 0.039$). The β_1 isoform in the cast leg after immobilisation was 26 % lower than in the non-cast leg ($p = 0.018$), but did not differ at 14 days recovery compared to either the non-cast leg or non-immobilised control group. The β_2 isoform in the cast leg after immobilisation was 71% lower than the non-cast leg ($p = 0.004$) and 65% lower than non-immobilised control group ($p = 0.012$), but did not differ at 14 days recovery, compared to either the non-cast leg or non-immobilised control group. There were no differences in the abundances of the α_1 and α_3 isoforms between legs or groups. Thus the [^{3}H]ouabain binding site content and α_2 were decreased with immobilisation, and remained depressed at 14 days recovery in the castration group, with the NKA α_2, β_1 and β_2 isoform abundances also decreased with immobilisation compared to sham group. The β_3 isoform abundance could not be detected in either sham or castration groups. Hence testosterone suppression was associated with impaired restoration of immobilisation-induced lowered NKA α_2 isoform and of the number of functional NKA in rat soleus muscle.

Study two. This study investigated which NKA isoforms (α_1 - α_3 and β_1 - β_3) accompanied an anticipated upregulation of skeletal muscle NKA content following sprint exercise training. Fifteen healthy young adults (11 males, 4 females) underwent either 7 weeks sprint training comprising repeated 30-s maximal sprint cycling efforts, three days/week ($n = 8$, ST) or a 7 wk control period ($n = 7$, CON). A vastus lateralis biopsy was taken at rest, prior to and following ST or CON and analysed for NKA content and NKA α_1 - α_2 and β_1 - β_2 isoform abundances. The muscle NKA content tended to increase after ST (28 %, $p = 0.063$, $d = 0.487$), with no changes in CON; six of the eight

participants responded to training with an increased NKA content. However, there were no significant changes with ST for any of NKA α_1, α_2, β_1, or β_2 isoform abundances. The α_3 and β_3 isoforms could not be detected. No sprint performance improvements occurred after ST (appendix 8). The unchanged [^{3}H]ouabain binding site content of the whole group was surprising but was consistent with unchanged abundances in the NKA isoforms. This likely reflects a Type II error, but might also reflect an inadequate training stimulus as consistent with the lack of performance improvement. Further research with a larger sample size is required to ascertain which isoforms are upregulated with increased NKA content with ST.

Study Three. This study investigated which NKA isoforms (α_1 - α_3 and β_1 - β_3) accompanied an anticipated increase in skeletal muscle NKA content after resistance exercise training. Twenty-one healthy young males underwent 7 weeks of resistance training (RT, n=16) or control period (CON, n=5). Participants underwent a vastus lateralis muscle biopsy at rest, prior to and following RT or CON, for measurement of NKA content and NKA α_1 - α_3, and β_1 - β_3 isoform abundances. After RT, the muscle NKA content increased by 12% ($p = 0.012$), NKA α_1 isoform increased by 32% ($p = 0.013$) and the α_2 isoform increased by 10% ($p = 0.001$), with no significant changes in CON. There were no differences in the β_1 or β_2 isoform abundances following RT. The α_3 and β_3 isoforms could not be detected. Thus the resistance training-induced increase in muscle NKA content was accompanied by increases in both the α_1 and α_2 NKA isoform abundances. It is speculated that specific adaptations accrued that function to both resist fatigue during intense resistance training sessions, as well as facilitate recovery after sets within training, in these healthy young adults.

Study four. This study investigated the effects of moderate intensity continuous (MICT) and of high intensity interval exercise training (HIIT), on each of muscle NKA content

and NKA α_1 - α_3 and β_1 - β_3 isoform abundances in patients with chronic kidney disease (CKD) and also explored possible differences in rest muscle in CKD compared against healthy participants. Fifteen patients (six females, nine males) underwent 12 weeks of either MICT (n = 5), or HIIT (n = 8) on a motorised treadmill, whilst 2 patients acted as controls. Fifteen healthy, age- and sex-matched participants acted as controls. The HIIT comprised four intervals of 4 min duration at 85 - 95% HR_{max}, with an intervening 3 min of active recovery at 65% HR_{max}, whilst MICT comprised 30 min training at 65% HR_{max}. A vastus lateralis muscle biopsy was taken at rest, prior to and post-training, for measurement of NKA content and NKA α_1 - α_3 and β_1 - β_3 isoform abundances. There were no significant differences between CKD and CON in rest muscle (n = 15) for NKA content ($p = 0.459$), α_1 ($p = 0.984$), α_2 ($p = 0.235$), β_1 ($p = 0.247$) or β_2 ($p = 0.138$) isoforms. No significant differences in muscle NKA content were found following MICT ($p = 0.165$) or HIIT ($p = 0.278$), and pooled data from both groups (n = 15) also revealed no increase after training ($p = 0.110$). No significant differences in NKA α_1, α_2, β_1 or β_2 isoform abundances were found following either MICT or HIIT, but there was a significant increase in α_2 in the pooled data after training ($p = 0.035$). The α_3 and β_3 isoforms could not be detected. Hence training protocols that might be anticipated to enhance muscle NKA content and NKA isoform abundances in healthy participants failed to do so in patients with CKD. The lack of increase in pooled data argues against a possible type II error. Further study is required to verify whether processes underlying upregulation in muscle NKA are impaired in CKD and whether upregulation is possible in CKD with different training protocols or training types such as resistance training.

In conclusion, this thesis demonstrated that hindlimb immobilisation reduced [^{3}H]ouabain binding site content in both sham and castration groups. The muscle NKA α_2, β_1 and β_2 isoform abundances were not reduced with hindlimb immobilisation in the sham, but were

reduced in the castration group. Importantly, this thesis demonstrated that castration impaired the recovery of [^{3}H]ouabain binding site content and muscle NKA β_2 isoform abundance after reductions with hindlimb immobilisation. This thesis revealed that in humans, resistance training was effective in increasing muscle NKA content and NKA α_1 and α_2 isoforms, whilst sprint training only tended to increase NKA content but without changes in any of NKA isoform abundances. This thesis also demonstrated that CKD had no effect on resting muscle NKA content or NKA isoform abundances compared to healthy controls, and furthermore, that both MICT and HIIT were unable to upregulate the NKA content and isoform abundances in patients with CKD.

Declaration

"I, *Muath Altarawneh*, declare that the Doctor of Philosophy dissertation entitled *'**Exercise Training, Immobilisation and Castration Effects on Skeletal Muscle Na^+,K^+-ATPase**'* is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices and bibliography references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for any other academic degree or diploma. Except where otherwise indicated this dissertation is my own work".

Signature	Date
Muath Altarawneh	November 2018

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This thesis involved multiple projects contributions with a number of different health researchers and professionals. I independently performed all of the muscle tissue analyses using the [^{3}H]ouabain content binding site and Western Blotting techniques, statistical analysis and wrote up thesis chapters. I was also heavily involved in the conduct of the sprint training study contributing to the experimental trials and blood analyses.

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Abbreviations

Muscle		**unit**
Na^+,K^+-ATPase	sodium-potassium adenosine 5'triphosphatase	
NKA	Na^+,K^+-ATPase	
ATP	adenosine 5' triphosphate	
t-tubules	tranverse tubules	
SOL	soleus muscle	
EDL	extensor digitorum longus muscle	
α	Alpha subunit	
β	Beta subunit	
[^{3}H]ouabain binding	tritiated ouabain binding	$(pmol.(g\ wet\ wt.)^{-1}$
3-O-MFPase	3-O-methylfluorescein phosphatase	$(nmol.(g\ wet\ wt.)^{-1}$
$pmol.g^{-1}$	picomoles per gram	
kDa	kilodalton	
µg	microgram	
Electrolytes		
K^+	potassium ion	
$[K^+]$	potassium ion concentration	mM
$[K^+]_i$	intracellular potassium ion concentration	
$[K^+]_e$	extracellular potassium ion concentration	
Na^+	sodium ion	
$[Na^+]$	sodium ion concentration	mM
$[Na^+]_i$	intracellular sodium ion concentration	
$[Na^+]_e$	extracellular sodium ion concentration	
Ca^{2+}	Calcium ion	
Aerobic power		
VO_2	oxygen consumption	
$VO_{2\ peak}$	peak oxygen consumption	$L.min^{-1}$
VO_{2max}	maximal oxygen consumption	$L.min^{-1}$

Publications and Presentations

The following manuscripts that have been prepared for submission.

Muath M. Altarawneh, Erik D. Hanson, Andrew C. Betik, Aaron C. Petersen, Alan Hayes, Michael J. McKenna. Effects of testosterone suppression, hind limb immobilization and recovery on [^{3}H]ouabain binding site content and Na^{+}, K^{+}-ATPase isoforms in rat soleus muscle. Submitted to Journal of Applied Physiology

Muath M. Altarawneh, Aaron Petersen, Trevor Farr, Andrew Garnham, James Broatch, Shona Halson, David Bishop1 and Michael J. McKenna Resistance training upregulates skeletal muscle Na^{+},K^{+}-ATPase content, with elevations in α_1 and α_2 but not β isoforms. To be submitted to European Journal of Applied Physiology.

Conference Presentations:

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Chapter 1. General introduction

The primary functions of skeletal muscle are to generate mechanical force and power, maintain posture, and control body movement via converting chemical energy into mechanical energy and thus contributing to body generation (Sarvazyan et al. 2014; Frontera and Ochala 2015). The ability of muscle to undergo contraction depends initially on membrane excitability due to large chemical gradients for sodium ($[Na^+]$) and potassium ($[K^+]$) ion concentrations across the sarcolemma (Nielsen and Clausen 2000). Reduced trans-sarcolemmal gradients of $[Na^+]$ and $[K^+]$ can result in a loss of cellular excitability and contribute to muscle fatigue (Sejersted and Sjøgaard 2000). In skeletal muscle, the sodium-potassium adenosine triphosphatase (Na^+,K^+-ATPase, NKA), also called the Na^+,K^+-pump, has a major regulatory function in maintaining the electrochemical gradients of $[Na^+]$ and $[K^+]$ across the cell at rest, as well as during and following muscle contractions (Clausen 2008; Kaplan 2002). Thus regulation of NKA is critical for cellular excitability and muscle function (Juel et al. 2013; Green 2004; Clausen 2013b). In skeletal muscle, the NKA comprises an α and β subunit each with multiple isoforms (α_1 - α_3) and (β_1 - β_3) (Murphy et al. 2004; Thomassen et al). The NKA in muscle can be downregulated and upregulated by a number of factors including inactivity, various hormones and physical activity (Therien and Blostein 2000; Ewart and Klip 1995; Clausen 2013b; Nielsen and Harrison 1998).

The broad aim of this thesis was to investigate several factors affecting the possible downregulation and upregulation of NKA in skeletal muscle. Study 1 (Chapter 3) investigated the possible downregulation of NKA after hindlimb immobilisation and testosterone suppression in rat skeletal muscle. Studies 2 - 4 (Chapters 4 - 6) investigated the possible upregulation of NKA after exercise training in human skeletal muscle. A

common theme across all four chapters was the influence of a change in physical activity through different interventions on muscle NKA. Hindlimb immobilisation for 1 - 4 weeks decreased NKA, as measured by [^{3}H]ouabain binding site content, in rat soleus muscle by 20 - 30% (Kjeldsen 1986; Zemkova et al. 1990), by 23 - 25% in guinea pig gastrocnemius muscle (Leivseth et al. 1992) and by 39% in sheep vastus lateralis muscle (Jebens et al. 1995) but without measures of specific isoforms. Hindlimb immobilisation in rat soleus muscle for 6 hours to 3 days reduced the NKA α_2 isoform "electrogenic activity" by 72% to 89%, with no change in α_1 isoform "electrogenic activity" (Kravtsova et al. 2015; Kravtsova et al. 2016). However, the effects of longer immobilisation on NKA isoforms and also on α_3, β_1, β_2 and β_3 are unknown. Importantly, study has investigated the effects of immobilisation using the combined measures of the [^{3}H]ouabain binding site content and NKA isoform abundances after hindlimb immobilisation.

Testosterone is a male sex hormone and plays a key role in muscle mass and strength (Leichtnam et al. 2006; Kvorning et al. 2006). A decline in testosterone is associated with a loss of muscle mass and strength (Sinha et al. 2014), but no study has yet investigated the possible effects of testosterone suppression on skeletal muscle NKA. Whether testosterone suppression via castration would reduce the skeletal muscle [^{3}H]ouabain binding site content and NKA isoform abundances particularly after immobilisation is unknown. Study 1 (Chapter 3) therfore investigated the effects of hindlimb immobilisation and testosterone suppression via castration on the [^{3}H]ouabain binding site content and NKA isoform protein abundances in rats soleus muscle.

Sprint training in humans comprising repeated 30 s maximal bouts, increased skeletal muscle NKA content by 8 - 16% (McKenna et al. 1993; Harmer et al. 2006). Sprint training comprising repeated 30 s bouts at 130%VO_{2max} elevated the NKA α_2 isoform by 68% and β_1 isoform (percentage not reported), but did not increase the α_1 isoform (Mohr

et al. 2007). In contrast, sprint training comprising repeated 30 s running bouts at 90 - 95% max running speed, increased the NKA α_1 isoform by 29%, but with no changes in α_2 and β_1 isoforms (Iaia et al. 2008). Neither study measured NKA α_3, β_2 and β_3 isoform abundances or NKA content. Hence measuring NKA α_3, β_2 and β_3 isoform abundances with NKA content is important to further understand the NKA adaptability in muscle after sprint training.

Resistance training in human increased muscle NKA content by 16% (Green et al. 1999b). In contrast, resistance training did not increase muscle NKA content in another study, although the NKA content was increased in data pooled from three separate trained groups by 15% (Medbø et al. 2001). A further study found that resistance training increased each of NKA α_1, α_2 and β_1 isoforms, by 37%, 21% and 33%, respectively (Dela et al. 2004), although the NKA α_3, β_2 and β_3 isoforms and NKA content were not investigated.

Thus it is unclear from both repeated 30 s sprint or resistance training studies which isoforms are associated with the adaptation of NKA content following training, since no study has combined measures of muscle NKA content and NKA isoform α_1 - α_3 and β_1 - β_3 abundances following training. Therefore, this thesis investigated the effects of each of sprint (Study 2, Chapter 3) and resistance exercise training (study 3, Chapter 4) on skeletal muscle NKA content combined with α and β isoform abundances in healthy young adults, to clarify which NKA isoforms were upregulated with an increase in skeletal muscle NKA content.

Patients with chronic kidney disease (CKD) exhibited impaired plasma K^+ regulation during incremental cycling exercise, which was suggested to contribute to muscle fatigue and reduced exercise performance (Petersen et al. 2009; Sangkabutra et al. 2003). Similarly, impaired exercise performance in patients with CKD was suggested to be due

to depressed maximal muscle NKA activity (Petersen et al. 2011). One study reported no differences in muscle NKA content and isoform abundances between patients with CKD and healthy participants (Petersen et al. 2011). Thus the effects of CKD on muscle NKA and K^+ regulation appear contradictory. Whilst several studies have shown that exercise training induced upregulation of skeletal muscle NKA content in healthy participants (Green et al. 2008; Green et al. 2004; Evertsen et al. 1997, Benziane et al. 2011), no study has investigated the effects of exercise training on muscle NKA in CKD. It is important to understand whether NKA is malleable with training in these patients and thus whether this may be important in enhancing exercise performance. Study 4 (Chapter 6) therefore investigated firstly if any differences existed between patients with CKD and healthy participants, in skeletal muscle NKA content and isoform abundances, and then investigated the effects of moderate and high intensity exercise training on skeletal muscle NKA content and isoform abundances in CKD.

Chapter 2. Literature Review

2.1 Muscle contraction, sodium and potassium ion concentrations

Muscle contractions result from action potential (AP) propagation along the plasma membrane and into transverse tubules (t-tubules), where the AP is detected by voltage sensors, known as dihydropyridine receptors. The interaction between the dihydropyridine receptors and the sarcoplasmic reticulum ryanodine receptor causes Ca^{2+} release from sarcoplasmic reticulum into the cell cytoplasm and consequent cross bridge interaction (Allen et al. 2008; Lamb 2000). Each AP comprises an influx of Na^+ ions into, and efflux of K^+ ions from the muscle cells, creating Na^+ and K^+ ion currents across the cell membrane. The intracellular $[K^+]$ ($[K^+]_i$) is maintained at around 140 - 165 mM by active K^+ pumping into the cell from the extracellular environment, where the $[K^+]$ ($[K^+]_e$) is around 5 mM. Through cellular extrusion of Na^+, the NKA constrains the rise in intracellular $[Na^+]$ ($[Na^+]$i) that would otherwise occur with repeated AP (Clausen 2010; Sejersted and Sjøgaard 2000). Hence, the NKA maintains a low intracellular $[Na^+]$ to $[K^+]$ ratio in the face of an inward concentration gradient for Na^+ and an outward gradient for K^+ and fluxes in both ions due to each AP (Mobasheri et al. 2000. Thus precise regulation of the NKA with muscle contractions to maintain cellular $[Na^+]$ and $[K^+]$ is needed.

2.1.1 The relationship between membrane potential and $[K^+]$

The resting membrane potential (Em) ranges between 75 - 89 mV, and allows muscle fibres to generate and propagate AP (McKenna et al. 2008; Sejersted and Sjøgaard 2000). Any decline in Em (depolarisation) resulting in membrane depolarisation induced inactivation of voltage Na^+ channels, impairs AP propagation and results in a loss of muscle excitability (Allen et al. 2008). In skeletal muscle K^+ plays a vital role in maintaining Em, thus any change in $[K^+]$ across the sarcolemma could potentially impact Em (Sejersted and Sjøgaard 2000).

Several studies have shown a correlation between the rise in $[K^+]_e$ and the decline of Em in muscle. In rats soleus muscle, high frequency stimulation at 100 Hz increased $[K^+]_e$ up to 14 mM, which induced a decrease in Em from -74 to -53 mV (Cairns et al. 1995). In mouse muscle, high frequency stimulation at 120 Hz in soleus and at 200 Hz in extensor digitorum longus (EDL) muscles, elevated $[K^+]_e$ from 7 to 14 mM and reduced Em by~35% in both soleus and EDL muscles (Fig 2.1) (Cairns et al. 1997). In rat EDL muscle, AP firing frequency at 6, 15 and 30 Hz increased $[K^+]$ in the t-tubular system from 5.3 mM to 6.8, 8.1 and 11.2 mM which was associated with a 0.97, 2.08 and 4.4 mV decrease in Em, respectively (Fraser et al. 2011). In human vastus lateralis muscle, as a results of changes in the $[K^+]_i$, $[K^+]_e$, $[Na^+]_i$ and $[Na^+]_e$, the Em was calculated to fall from -83 to -75 mV following intense knee extensor exercise (Sjogaard et al. 1985). These calculations however, underestimated the rise in $[K^+]_e$ in muscle.

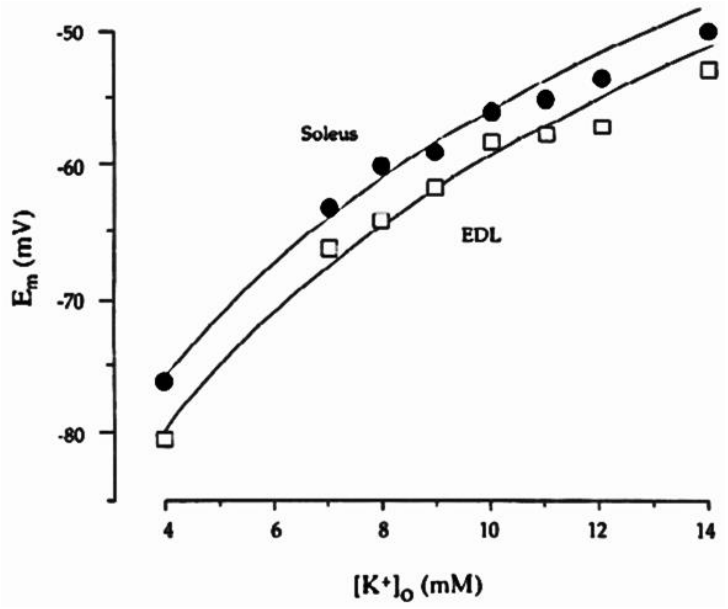

Figure 2.1. Resting membrane potential (Em)-$[K^+]$ relationship for soleus (●) and EDL (□) mucles.

Each point is mean Em, value of impalements in 43 fibres from 4 soleus muscles and 131 fibres from 13 EDL muscles obtained under steady state conditions. (Cairns et al. 1997).

2.1.2 Effects of elevated of muscle $[K^+]_e$ and reduced $[K^+]_i$ on muscle fatigue

During muscle contractions, muscle $[K^+]_e$ increased up to 10 - 12 mM, which was proposed to lead to fatigue development (Nordsborg et al. 2003; Nielsen et al. 2004a). Several in-vitro studies have demonstrated that elevated $[K^+]_e$ caused a reduction in tetanic force. In isolated rat soleus muscle, increased extracellular $[K^+]_e$ from 4 to 10 mM resulted in a 40% reduction in twitch and tetanic force, whilst increased $[K^+]_e$ to 12.5 mM reduced tetanic force by 95% (Clausen et al. 1993). Similarly, in isolated rat soleus muscle, an increase in $[K^+]_e$ from 4 to 11 mM caused an 85% reduction in tetanic force (Pedersen et al. 2005). In isolated mouse soleus muscle, an increase in $[K^+]_e$ from 5 to 10 mM induced a 40% reduction in tetanic force (Juel 1988). In frog sartorius muscle, elevated $[K^+]_e$ from 3 to 7 mM decreased tetanic force by 41% (Bouclin et al. 1995).

In human vastus lateralis muscle, $[K]_i$ declined from 161 to 141 mM (Sjogaard 1983) and from 165 to 129 mM during repeated muscle contractions (Sjogaard et al. 1985), which was suggested to contribute to muscle fatigue (Sjogaard 1983). In skinned fibres from rat EDL muscle, $[K]_i$ decreased from 113 to 60 mM and resulted in a 30% decline in twitch force (Nielsen et al. 2004b). During 40 Hz stimulation, $[K]_i$ decreased by 32 mM in rat soleus muscle and by 48 mM in EDL muscle, which was associated with 29 and 10 % reduction, respectively, in muscle force (Juel 1986). These findings have demonstrated that increased $[K^+]_e$ during muscle contraction may play an important role in muscle fatigue (Juel et al. 2000; Fitts 1994). However, changes in chloride (Cl^-) ion conductance has been shown to contribute to maintaining Em and muscle excitability which might protected against the depolarising effect of $[K^+]$ (Pedersen et al. 2005; Cairns et al. 2004; Dutka et al. 2008). In rat EDL muscle, excitation leads to a rapid efflux of $[K^+]$ and resulting in loss of muscle excitability, whilst the influx of Cl^- contributes to repolarisation via clearance of K^+ via inward rectifier K^+ channels (Clausen 2013a). Loss

of muscle tetanic force as a result of increased $[K^+]_e$ in rat soleus muscle was recovered via reducing Cl^- conductance (Pedersen et al. 2005). This relationship between $[K^+]$, Cl^- conductance and Em is still unknown in human skeletal muscle.

2.1.3 $[Na^+]$ and muscle fatigue

In rat isolated soleus and EDL muscles, a reduction in $[Na^+]_e$ from 147 to 30 mM and 25 mM decreased both twitch and tetanic force by ~55% (Cairns et al. 2003). Similarly, decreased $[Na^+]_e$ from 147 to 25 mM reduced tetanic force by ~66% (Overgaard et al. 1997). In skinned fibres from rat EDL muscle, elevated $[Na^+]_i$ from 20 mM to 50 mM reduced the ability of the t-tubules to support AP (Nielsen et al. 2004b). In rat isolated soleus muscle, there was a significant negative correlation between increased $[Na^+]_i$ and reduced muscle force during 60 Hz stimulation (Nielsen and Clausen 1996). In frog semitendinosus muscle, increased $[Na^+]_i$ from 16 to 49 mM was associated with decrease in Em from -38 to -74 mV (Balog and Fitts 1996).

The results from these studies have demonstrated that changes in muscle intracellular and extracellular $[Na^+]$ and $[K^+]$ have been implicated as a potential cause of muscle fatigue during muscle contractions via changes in EM. Given that NKA pays a major role in THE regulation of both $[Na^+]$ and $[K^+]$ gradients across the cell, it is likely that NKA have a primary role in skeletal attenuating muscle fatigue. Therefore, this literature review focuses on NKA regulation in skeletal muscle.

2.2 Sodium-Potassium Adenosine Triphosphatase (Na^+,K^+-ATPase, NKA) in skeletal muscle

2.2.1 Definition and function of NKA

The NKA (Fig 2.2) is a membrane-associated protein complex that is ubiquitously expressed in most eukaryotic cells (Vague et al. 2004; Clausen 2013b; Clausen et al. 2017). The NKA was first identified in 1957 by Skou (Skou 1957) for which he was awarded the Nobel prize for chemistry (Clausen 1998). In skeletal muscle, the NKA are thought to be primarily localised in the sarcolemma and the t-tubules (Clausen 2003a). The NKA produces and maintains steep transmembrane [Na^+] and [K^+] gradients via cellular uptake of two K^+ and extrusion of three Na^+ ions, whilst hydrolyzing ATP generated from cellular glycolysis (Nyblom et al. 2013; Reinhard et al. 2013). The NKA mediated Na^+/K^+ exchange is electrogenic and thus NKA activity contributes to membrane hyperpolarisation, which is essential for muscle excitability (Clausen 1996b). In addition to this ion-pumping function, the NKA is also involved in complex intracellular signalling. This may occur via direct protein-protein interactions between NKA and its neighbouring proteins, triggering a signalling cascade, culminating in gene transcription (Aperia et al. 2016; Reinhard et al. 2013).

2.2.2 Structure of NKA

The NKA comprises four α subunit isoforms (α_1 - α_4) and three β subunit isoforms (β_1 - β_3), with both α and β subunits required for NKA function (Fig 2.2). A third subunit is a member of the FXYD family (Kaplan 2002; Bibert et al. 2008).

2.2.2.1 The α subunit isoforms

The α subunit spans the membrane ten times forming trans-membrane domains (M1 to M10), with both the N- and C-termini being localised on the cytosolic side. The α subunit is composed of about 1000 amino acids and exhibits a molecular mass of 100 to 113 kDa

and is responsible for the catalytic processes of the enzyme comprising the binding of Na^+ and K^+ ions and their transport across the membrane, fuelled by the hydrolysis of ATP (Scheiner-Bobis 2002; Clausen 2003a).

In human skeletal muscle, all NKA isoforms except α_4 are found (Murphy et al. 2004). In human soleus muscle, the α_1 isoform was found to be located in the plasma membrane, with only around 4% located in an intracellular membrane fraction (Hundal et al. 1994). The α_2 isoform was mainly detected in the plasma membrane, and around 75% in intracellular membrane fractions, while the α_3 isoform was also mainly located in the plasma membrane (Hundal et al. 1994). The α_1 isoform was reported to be equally distributed in type I and type II single muscle fibres, whereas the α_2 isoform had a 37% higher abundance in type II than type I fibres in human muscle biopsy samples (Thomassen et al. 2013). A subsequent study in human single muscle fibres found both the α_1 and α_3 isoforms to be similarly expressed in type I and II fibres (Wyckelsma et al. 2015), and in contrast to Thomassen et al (2013) also found no α_2 isoform differences between fibre types (Wyckelsma et al. 2015).

In rat skeletal muscle, the α_1 isoform was detected in both soleus (SOL) and extensor digitorum longus (EDL) muscles and was present in sarcolemma and in t-tubules (Kristensen and Juel 2010). The α_1 had a higher abundance in oxidative than in glycolytic muscles in the rat (Kristensen and Juel 2010; Fowles et al. 2004; Thompson and McDonough 1996). In contrast, the α_2 was equally distributed in oxidative and glycolytic muscles (Fowles et al. 2004; Juel et al. 2001; Thompson and McDonough 1996). In murine skeletal muscle, both the α_1 and α_2 isoforms were present in the sarcolemma (Williams et al. 2001).

The α_1 isoform appears to play a "cellular housekeeping role" regulating $[Na^+]$ and $[K^+]$ trans-membrane gradients in resting muscle, given a high affinity for both Na^+ and K^+

ions and considering the higher abundance of α_1 in the sarcolemma (Crambert et al. 2000). The α_2 plays a major role in Na^+ and K^+ transport in working muscle. The α_2 has a lower affinity for K^+ than α_1 in resting muscle (DiFranco et al. 2015), and the α_2 activity increased rapidly in contracting muscle in response to increased $[K^+]_e$ (DiFranco et al. 2015). Research using gene knockout techniques in muscle show the importance of α_1 and α_2 isoforms. In mice, partial global knockout of the α_1 isoform reduced contractile force in EDL muscle (Lingrel et al. 2003). The complete global knockout in mice of the α_2 isoform caused the animal to either be borne dead or to die shortly after birth (Lingrel et al. 2003). In mice, skeletal muscle specific α_2 knockout reduced each of muscle strength, endurance and exercise tolerance, suggesting that the α_2 plays a significant role in maintaining contractions and resisting fatigue in skeletal muscle (Radzyukevich et al. 2013). This was also despite an upregulation of the α_1 isoform (Radzyukevich et al. 2013). The α_3 isoform is predominantly expressed in neurones (Matchkov and Krivoi 2016; Clausen et al. 2017; Bøttger et al. 2011). It was earlier suggested that the α_3 isoform could be activated and work as a spare isoform to help restore membrane potential, when the Na^+ and K^+ fluxes were higher and with NKA α_1 and α_2 working at maximal rates during depolarization and repeated action potentials (Blanco & Mercer, 1998). However, the exact role of the α_3 isoform in skeletal muscle, is unclear, and further research is required to determine the functional of NKA α_3 in skeletal muscle.

2.2.2.2 The β subunit isoforms

The β subunit spans the membrane once, with the N-terminus localised on the intracellular side of the membrane (Reinhard et al. 2013). The β subunit is glycosylated, and is composed of about 370 amino acids (Kaplan 2002) exhibiting a molecular mass of about 40 to 60 kDa (Mobasheri et al. 2000; Kaplan 2002), which varies for the β_1, β_2 or β_3 isoforms (Cougnon et al. 2002; Blanco and Mercer 1998). The β subunit is responsible

for regulating NKA activity and also transporting and stabilising of movement of the α subunit from the endoplasmic reticulum to the plasma membrane (Hundal et al. 1994).

Each of the β_1, β_2, and β_3 isoforms have been detected in rat (Arystarkhova and Sweadner 1997; Ng et al. 2003) and in human skeletal muscle. In humans, the β_1 and β_3 isoforms were found to be similarly expressed in both type I and II fibres (Wyckelsma et al. 2015), whereas the β_2 was 27% more abundant in type II fibres (Wyckelsma et al. 2015; Thomassen et al. 2013). The β_1 was detected mainly in the plasma membrane with a small amount being found in the intracellular fraction (Hundal et al. 1994). In the rat, the β_1 isoform has a higher abundance in muscles comprised predominantly of slow twitch fibres, while the β_2 has a higher abundance in muscles comprised predominantly of fast twitch fibres (Fowles et al. 2004; Zhang et al. 2006). The β_3 isoform was found to be similarly abundant in rat red and white gastrocnemius muscles (Ng et al. 2003). The β_1 and β_2 isoforms were detected in the sarcolemma and t-tubules in rat skeletal muscle (Hundal et al. 1994).

Whilst it is known that the β-subunit is essential for the transport of the α-subunit to the plasma membrane (Geering 1990; Mcdonough et al. 1990; Chow and Forte 1995), the function of each of the NKA β isoforms is still unclear. However, in basal conditions, the β_1 isoform shows a greater affinity to Na^+ and lower affinity to K^+ compared to β_2 (Crambert et al. 2000). Unlike with the α isoforms, no research on β isoforms knockout animal models has yet been conducted.

2.2.2.3 The phospholemman subunit

Phospholemman (PLM), also called FXYD1 is a member of the FXYD family, which is expressed as seven isoforms (FXYD 1 - 7) (Bibert et al. 2011; Geering 2006). The PLM is an additional accessory protein for the NKA regulatory subunit (Fig 2.2). It mainly associates with NKA α_1 and α_2 isoforms, exerts an inhibitory effect on NKA activity and

has a relative mass of only 7 - 11 kDa, and is primarily expressed in skeletal muscle (Li et al. 2004; Mishra et al. 2011; Geering 2008).

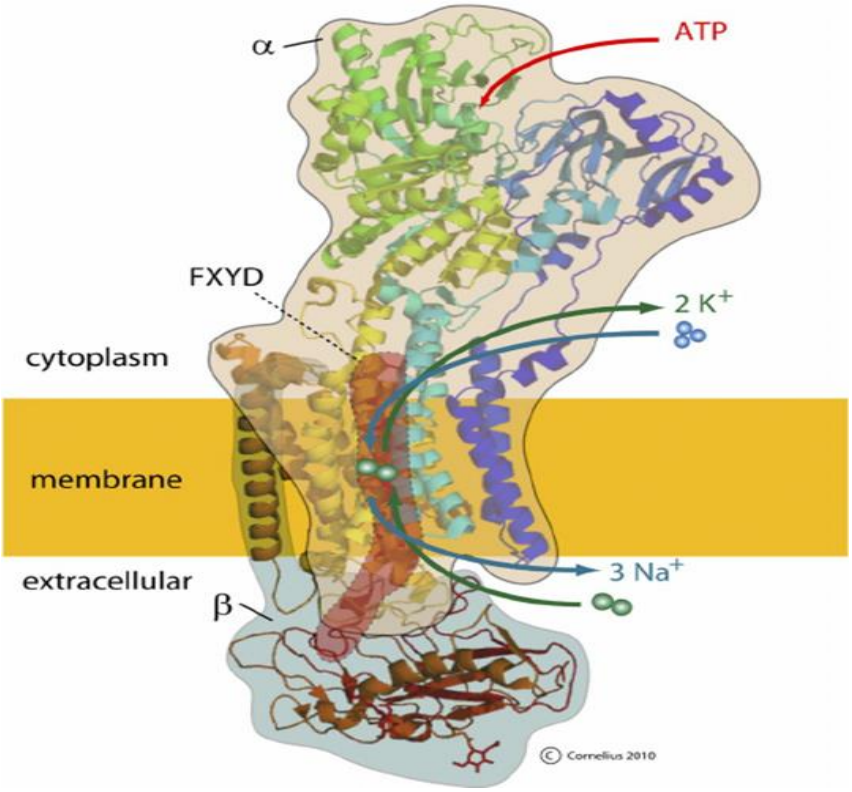

Figure 2.2. Molecular structure of Na^+ , K^+ -ATPase.
(Morth et al. 2007), drawn by Flemming Cornelius

2.2.2.4 [^{3}H]ouabain binding site content

The [^{3}H]ouabain binding site content technique is the traditional method used to measure NKA content in skeletal muscle, being based on the high binding affinity of cardiac glycosides to the NKA α subunit with a stoichiometry of 1:1 (Hansen 1984). The NKA α_1 isoform has a low affinity to cardiac glycosides in rat muscle, thus the standard [^{3}H]ouabain binding site content technique is unable to detect the α_1 isoform (Clausen 2003a; Hansen 2001). In rat EDL and soleus muscles, the α_1 isoform represents around 15 - 25% of the total pool of NKA (Hansen 2001). Another study in rat EDL muscle showed that, the NKA α_1 isoform only accounts for 13% whilst the α_2 isoform comprised 87% of the total α isoforms; however, the NKA α_3 isoform was not detected (He et al. 2001). Thus, in rat skeletal muscle, the [^{3}H]ouabain site content assay does not detect all α subunits. In contrast, in human skeletal muscle, the NKA α_1, α_2, and α_3 isoforms each have a similar ouabain affinity and thus all are detected using the [^{3}H]ouabain binding site content method (Wang et al. 2001). Therefore, in human skeletal muscle, the [^{3}H]ouabain binding site content is a quantitative measure of and can also be referred to as the NKA content, whereas in rodent skeletal muscle this is better expressed just as [^{3}H]ouabain binding site content (Nørgaard 1986; Nørgaard et al. 1984). Therefore, in this thesis these measures will be described as [^{3}H]ouabain binding site content in study one which utilised rat skeletal muscle and thereafter as NKA content in Studies two, three and four, which all utilised human skeletal muscle. The typical NKA content, measured as [^{3}H]ouabain binding site content, reported in human skeletal muscle is shown in Table 2.1.

2.2.2.5 Comparisons of muscle NKA variables in human and rat skeletal muscle

The key comparisons in skeletal muscle NKA between human and rat, as detailed in previous sections are as follows:

1. In human skeletal muscle, the NKA α_1, α_2, and α_3 isoforms each have a similar ouabain affinity, thus the [^{3}H]ouabain site content reflects the whole NKA content. In rat muscle, the [^{3}H]ouabain site content reflects the α_2 content due to lower of α_1 isoform affinity for [^{3}H]ouabain.
2. In both human and rat muscle, the NKA α_1 isoform is expressed in the sarcolemma while the α_2 isoform is present predominantly in the intracellular membrane fraction.
3. In human, the α_1 α_2 and α_3 are all ~equally abundant in both type I and type II fibres. In rat muscle, the α_1 is more abundant in oxidative than in glycolytic muscle, and the α_2 is similarly abundant in oxidative and glycolytic muscles.
4. In human muscle, the β_1 and β_3 isoforms are similarly expressed in both type I and II fibres, whereas the β_2 more abundant in type II fibres. In rat, the β_1 isoform has a higher abundance in type I fibre, and the β_1 isoform has a higher abundance in type II fibre.

Table 2.1. Vastus lateralis muscle [^{3}H]ouabain binding site content (NKA content) measured in muscle biopsy samples in healthy human males and females aged between 18 - 69 years.

Study	Age (Mean yr)	Number (sex M/F)	[^{3}H]-ouabain site content (pmol.g wet wt^{-1})
Klitgaard et al., 1989	68	6 M	237
Klitgaard et al., 1989	28	6 M	278
Green et al., 1993	19	9 M	339
McKenna et al., 1993	19	6 M	333
Madsen et al., 1994	30	39 M	307
Evertsen et al., 1997	18	11 M	343
Green et al., 1999a	21	16 M	284
Medbø et al., 2001	19	8 M	356
Fraser et al., 2002	26	8 M	311
Leppik et al., 2004	27	7 M 1 F	333
Petersen et al., 2005	24	8 M 7 F	291
Aughey et al., 2007	31	11 M	355
McKenna et al., 2012	24	8 M 8 F	350
McKenna et al., 2012	67	9 M 8 F	352
Perry et al., 2013	69	9 M 10 F	357
Wyckelsma et a l., 2017	69	9 M 6 F	373 344

2.2.2.6 NKA activity

The activity of NKA in skeletal muscle at rest is low, but increases rapidly during muscle contractions (Nielsen and Harrison 1998; Clausen 2008). At rest, the NKA activity is only at 2 - 6% of its maximal theoretical capacity, but when the isolated muscle was loaded with Na^+, reached up to 90% of its maximal activity (Clausen et al., 1987). The maximal activity of NKA has been measured in-vitro in human skeletal muscle by the 3-O-methylfeuorescein phosphatase (3-O-MFP) activity assay (Fraser and McKenna 1998) and impairments linked with fatigue (McKenna et al 2008). Submaximal cycling at ~75% $VO_{2\ peak}$ until fatigue reduced maximal in-vitro 3-O-MFP by ~11 - 13 % (Leppik et al. 2004) and incremental cycling to fatigue reduced 3-O-MFP by ~30% (Sandiford et al. 2005). Isometric exercise on an isokinetic dynamometer caused a reduction in NKA activity by ~35% (Fowles et al. 2002) and isokinetic contractions decreased 3-O-MFP by ~11 - 14% (Fraser et al. 2002; Petersen et al. 2005). Submaximal cycle ergometer exercise caused a 12% decrease in 3-O-MFP (Aughey et al. 2005). The 3-O-MFP assay has several disadvantages in measuring NKA activity after exercise, since it does not involve the hydrolysis of ATP, and relies on the K^+ stimulation without the Na^+ sensitivity (Juel et al. 2013). Given the role of phospholemman (PLM) phosphorylation in increasing the NKA activity via Na^+ activation, this assay thus may not reflect the activity of NKA caused by phospholemman with exercise.

A more recent technique used to quantify the NKA activity, measures phosphate (Pi) production from hydrolysis of ATP and Na^+-dependent activation (Juel et al. 2013). However, this assay uses a small fraction of the total protein, with a recovery of only 2% of total protein and thus may not reflect the whole muscle pool of NKA (Juel et al. 2013). Juel et al. (2013) showed no change after exercise in the Na^+-dependent activity but a reduction in 3-O-MFP activity, suggesting that the 3-O-MFP assay was not valid in

detecting changes in NKA activity with exercise. However, Hostrup et al (2014) reported a reduction in Na^+-dependent activity after exercise (Hostrup et al. 2014). Due to methodological concerns regarding both the 3-O-MFP and the ATP hydrolysis method utilised by Juel et al (2013), the activity of NKA in skeletal muscle was not measured in this thesis.

2.3 Effects of Age on skeletal muscle NKA

Few studies have investigated the effects of aging on skeletal muscle NKA in humans, with findings inconclusive. No significant difference was found in vastus lateralis muscle NKA content between older (mean age 68 years) and young, untrained (mean age 28 years) participants (Klitgaard and Clausen 1989). No differences were also detected in NKA content between young versus older (mean age 23.9 vs 66.8 years) participants (McKenna et al. 2012), or in 20 participants ranging from 25 - 80 years old (Nørgaard et al. 1984). In contrast, the NKA content in older adults aged between 69 - 81 years was 25.5% lower than those aged 55 - 68 years (Perry et al. 2013), however, this might be due to their chronic physical activity levels rather than their age (Wyckelsma and McKenna 2016). There was no difference in muscle NKA content between aged and young adults (mean age 25.5 vs 69.4 years) (Wyckelsma et al. 2016). Subsequent pooled analyses found that no age effect on NKA between younger adults aged 18 - 30 years compared to those aged from 55 to76 years (Wyckelsma and McKenna 2016) (Fig 2.4). Thus the evidences points no age effect on NKA.

The NKA isoform abundances were first reported lower by 24% for α_2 and 23% for β_3 isoform in older (mean age 66 years) than young adults (mean age 29 years), with no differences in other NKA isoforms (α_1, α_2, β_1 and β_2) (McKenna et al. 2012). However, when Wyckelsma et al (2016) normalized NKA isoform abundances against total protein rather than GAPDH protein as previously used (McKenna et al. 2012), since GAPDH

protein is highly expressed in Type II than Type I fibers, no differences were found in any of NKA isoform abundances between young and old adults (Wyckelsma et al. 2016). When NKA isoforms were measured in muscle single fibres, the NKA α_1 had a ~71% greater abundance in aged muscle in Type I fibre compared to young. The NKA α_3 and β_2 isoforms in aged muscle were both lower by ~47% and ~85%, respectively, in Type II fibers compared to young (Wyckelsma et al. 2016). The NKA β_3 was greater in aged muscle in both Type I and II fibers by ~96% and ~285%, respectively compared to young (Wyckelsma et al. 2016).

In animal models, clearer age effects have been reported. The muscle [^{3}H]ouabain binding site content from 85 day old rats was 58% lower in soleus (SOL) and extensor digitorum longus (EDL) muscles, when compared to infant rats (Clausen et al. 1982). In female guinea pigs, the [^{3}H]ouabain binding site content was 60% lower in EDL muscle from birth compared to maturity (Kjeldsen et al. 1984b). In rat soleus muscle, the [^{3}H]ouabain binding site content increased from 120 to 580 pmol.g wet wt^{-1} from 2 to 28 days of life, and then decreased to150 - 200 pmol.g wet wt^{-1} from 4 to 21 weeks of life (Kjeldsen et al. 1982). No difference was found in the [^{3}H]ouabain binding site content in either SOL or EDL muscles between 3 and 14 week old rats (Abdel-Azia et al. 1985). The α_2 isoform was lower by 30 - 40% in red and white gastrocnemius muscle in rats aged 18 and 30 months compared to 6 months (Sun et al. 1999). However, in SOL muscle, the α_1 isoform abundances increased by ~100% in one month old rats compared to those aged 6 months (Sun et al. 1999). In white and red gastrocnemius muscle, the α_1, β_1, and β_3 isoforms were more abundant, while the α_2 and β_2 isoform abundances decreased in 30-month compared to 6 month old rats (Zhang et al. 2006). Table. 2.2 presents a summary of finding on the effects of aging on NKA isoform abundances in human and rat skeletal muscle.

In summary, whereas studies in rat muscle showed that aging is associated with a decline in [^{3}H]ouabain binding site content and NKA isoform abundances, no clear age effects are seen in human skeletal muscle.

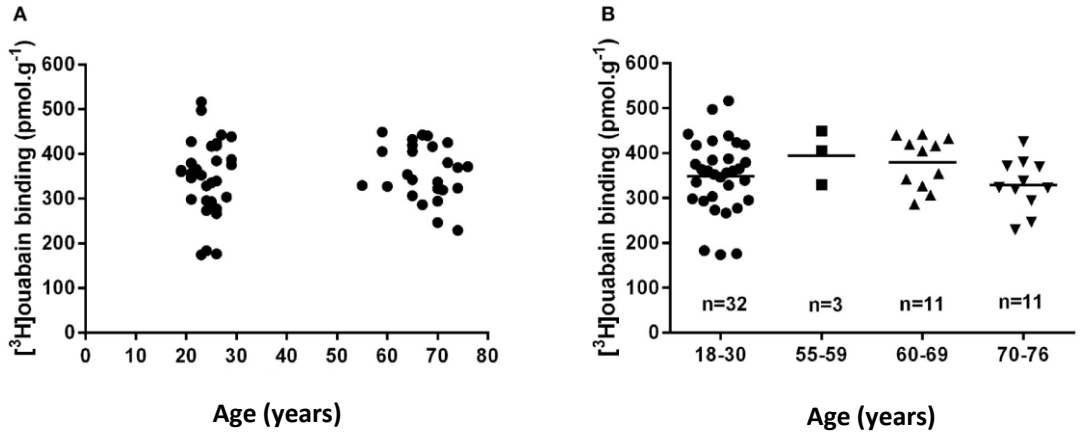

Figure 2. 3. Effects of aging on muscle [^{3}H]ouabain binding in human skeletal muscle.

Muscle [^{3}H]ouabain binding site content was collated from data collected on healthy young and healthy older adults from the McKenna research group between 2012 and 2016. (A) Shows the data combined into two discrete age groups, with no signifecant difference analysed by unpaired t-test ($p = 0.53$). (B) Shows all data plotted into relevant decades of life, analysed with no signifecant difference by one-way ANOVA ($p = 0.30$); the mean of each group is also shown as a horizontal line (From Wyckelsma and McKenna 2016).

Table 2.2 Effects of age on NKA isoform abundances in rat and human skeletal muscle

Ref	Species	Age (months/years)	N	Technique normalization	Muscle Measured	NKA Isoforms					
						α_1	α_2	α_3	β_1	β_2	β_3
1	Rat	Young (6 months)	5	Western blot	EDL	30 > 6	18 < 6				
		Adult (18 months)	6		Sol	30 > 6,18	18, 30 < 6		30 > 6,18	30 < 6	
		Old	7		RG	30 > 6,18	18, 30 < 6		30 > 6,18	30,18< 6	
		(30 months)			WG					30 < 18	
2	Rat	Young	NR	Western blot	EDL	NS	NS		NS		
		(16 months)			Sol						
		Old			RG	↑	NS		NS	↓	↑
		(29 months)			WG	↑	NS		NS	↓	↑
3	Rat	Young	12-	IHC	RG	↑ *	↓ *		↑ *	↓ *	↑ *
		(6 months)	15		WG	↑ *	↓ *		↑ *	↓*	↑ *
		Old (30 months)									

Table 2.2 continued

Ref	Species	Age (months/years)	N	Technique normalization	Muscle Measured	NKA Isoforms α_1	α_2	α_3	β_1	β_2	β_3
4	Human	Old 66.8 ± 6.4	17	Western blot (GAPDH)	VL	–	↓ 24%	–	–	–	–
		Young 23.9 ± 2.2	16								
5	Human	Old 69.4 ± 3.5	17	Western blot (Calibration Curve)	VL	–	–	–	–	–	↑ 250%
		Young 25.5 ± 2.8	14		VL Type I fibres	↑ 71%	–	–	–	–	↑ 96%
					VL Type II fibres	–	–	↓ 47%	–	↓ 85%	↑ 285%

Reference 1, Sun et al., 1999; 2, Ng et al., 2003; 3, Zhang et al., 2006; 4, McKenna et al., 2012; 5, Wyckelsma et al., 2016. Age, mean ± SD. Symbols: ↓, denotes decrease; –, no change; ↑, increase, data in parentheses denotes % difference between groups. ↑ * not quantitative but increased compared to young ↓ * not quantitative but decreased compared to young. NR not reported, NS not significant. Muscles: EDL, Extensor Digitorum Longus; Sol, Soleus; RG, Red Gastrocnemius; WG, White Gastrocnemius; VL, Vastus Lateralis. **(From Wyckelsma and McKenna 2016).**

2.4 Effects of selected hormones and sex differences on skeletal muscle NKA

2.4.1 Hormonal regulation

Numerous hormones regulate skeletal muscle NKA and further detail can be found in earlier major reviews (Clausen 2003a; Ewart and Klip 1995). Thyroid hormones, glucocorticoids and insulin all have a marked effect on NKA content and thus are briefly reviewed here.

2.4.1.1 Thyroid hormones

Thyroid hormones exert a major influence on NKA regulation. In humans, the NKA muscle content was 50% lower in patients with hypothyroidism and 68% greater in patients with hyperthyroidism, compared to euthyroid controls (Kjeldsen et al. 1984a) . The muscle NKA content was also reported to be 89% higher in hyperthyroid patients compared to euthyroid control (Riis et al. 2005) . In hyperthyroid rats, injection of a single dose of triiodothyronine (T3, 5-20 ug/100g body wt) increased the [^{3}H]ouabain site binding site content in SOL, gastrocnemius and EDL muscles by 9.8-, 5.1-, and 2.6-fold, respectively, compared to the hyporthyroid rat (Kjeldsen et al. 1986a). In isolated rat SOL muscle, injection of a single dose of triiodothyronine (T3, 20 ug/100g body wt) for 8 d increased the [^{3}H]ouabain binding site content by 103% (Everts and Clausen 1988).

2.4.1.2 Glucocorticoids

Glucocorticoids have been associated with upregulation of NKA content in skeletal muscle. In human skeletal muscle, ingestion of dexamethasone for 5 days (2 mg twice daily) respectively, increased NKA content by 18 and 24% and maximal 3-O-MFPase activity by 14 and 18% in the vastus lateralis and deltoid muscles (Nordsborg et al. 2005). In healthy males, ingestion of dexamethasone for 5 days (2 mg twice daily) increased NKA α_1 and α_2 isoforms by 17%, and increased β_1 and β_2 isoforms by ~6% and 8%,

respectively, compared to placebo (Nordsborg et al. 2008). In rat skeletal muscle, treatment with dexamethasone increased the [^{3}H]-ouabain site binding site content by 23–52% in EDL, soleus, gastrocnemius and diaphragm muscles (Dørup 1996).

2.4.1.3 Insulin

Insulin regulates the NKA in skeletal muscle acutely via increasing the affinity for intracellular Na^+ (Sweeney and Klip 1998; Pirkmajer and Chibalin 2016; Kitasato et al. 1980; Lytton 1985) Insulin also increased NKA content. In human patients with diabetes, the vastus lateralis muscle NKA content was 17 and 22% greater in patients with treated non-insulin-dependent diabetes mellitus and insulin-dependent diabetes mellitus than in control participants (Schmidt et al. 1994). Insulin increased the [^{3}H] ouabain binding site content by ~90% after 60 min of insulin incubation in plasma membrane fraction of frog skeletal muscle (Omatsu-Kanbe and Kitasato 1990), although the methodology was later criticised (Clausen 2003a). In untreated diabetic rats, lower insulin was associated with an 18% reduction in the [^{3}H] ouabain binding site content, conversely, after treatment with insulin, the level of [^{3}H]ouabain binding site content increased by ~23% (Schmidt et al. 1994).

2.5 Effects of sex differences and sex hormones (testosterone and estradiol) on skeletal muscle NKA

Few studies have examined the possible effects of sex differences on NKA content in skeletal muscle. No sex differences were reported in young sedentary through to old adults aged between 25 to 80 years (Nørgaard 1986), or between older males and females aged from 53 to 61 years (Green et al. 2001). Similarly, in older participants of mean age 69 years, no differences in NKA content were found between males and females (Wyckelsma et al. 2017). No sex differences in NKA content were found between young

recreationally active males and females (Murphy et al. 2007). In contrast, in elite skiers, the NKA content was found to be 18% higher in young adult males than females (Evertsen et al. 1997). These differences seen in elite level athletes may be result of training, whilst the participants in the other studies were untrained. Whilst findings from these studies suggest that sex difference is not associated with differences in [^{3}H]ouabain binding site content, further research is required to verify this and also to determine the possibility of sex differences on NKA isoform abundances.

2.5.1 Estradiol effects

Estradiol is the major estrogen hormone in women and produced by the ovaries, and has a vital role in reproductive cycle (Cohen et al. 2003), brain development and function (Vermeulen et al. 2002), lean mass (Brown 2008) and stimulating muscle damage repair via activation and proliferation of satellite cells (Enns and Tiidus 2010). No studies appear to have investigated estradiol effects on NKA in skeletal muscle. However, several studies have investigated the effects of estradiol therapy on NKA in cardiac muscle in rats. Injection of 40 mg.kg^{-1} estradiol for 24 hours was found to stimulate NKA activity in heart sarcolemma by 360% (Džurba et al. 1997; Obradovic et al. 2015). A dose of 0.5 mg.kg^{-1} of estradiol delivered via injection for 3 days increased the NKA activity in heart sarcolemma by 85% (Obradovic et al. 2015). However, an acute 40μg.kg^{-1} dose of estradiol for only 20, 30 and 40 min before analysis had no effect on the cardiac plasma membrane NKA α_2 isoform abundance (Koricanac et al. 2011).

2.5.2 Testosterone effects

Testosterone is a sex hormone present in males and has important biological effects, including of muscle hypertrophy, stimulation of protein synthesis and inhibition of protein degradation (Vingren et al. 2010), and is needed for bone growth and body development (Kumar et al. 2010; Brown 2008). Aging results in decreased testosterone

concentration, which can also occur as a result of different clinical conditions such as trauma and obesity; this decrease is associated with a decline in muscle mass and strength (Leichtnam et al. 2006; Brown 2008). Testosterone reduction can also occur as result of androgen deprivation therapy in prostate cancer patients (Mostaghel et al. 2007; Sharifi et al. 2005). Few studies have investigated the effects of testosterone on NKA. In rats, testosterone reduction via castration resulted in a 47 -73% decrease in NKA activity in rat erythrocyte membranes compared to a sham group (Sheng-qi et al. 1994). Injection of 50 µg testosterone for 2 days in castrated rats increased NKA activity 4-fold in the cerebral cortex compared to a sham group (Guerra et al. 1987). No studies have investigated the impacts of testosterone reduction on skeletal muscle NKA content, or on NKA isoform abundances. Given the importance of NKA for muscle function, it is beneficial to understand the impact of testosterone on NKA content and isoform abundances, with important implications particularly for people who suffer from lower testosterone. Therefore, the first study in this thesis investigated the effects of suppression in testosterone due to castration on skeletal muscle [^{3}H]ouabain binding site content and NKA isoform abundance in rat soleus muscle.

2.6 Intracellular [Na^+] regulation and NKA in skeletal muscle

Elevated $[Na^+]_i$ occurs with acute contractions (exercise), is one of the vital regulators for NKA in skeletal muscle, including possible mediator of NKA upregulation (Nielsen and Clausen 1997; Clausen 1996a; Sejersted and Hallén 1987). The maximal activity of NKA in an isolated soleus muscle was increased up to 90% after being loaded with Na^+ (Clausen and Flatman 1987). Following 30 s stimulation in isolated rat soleus muscle, the $[Na^+]_i$ increased by 22-fold, and resulted in a rapid increase in NKA activity, by 5 - 18 fold (Nielsen and Clausen 1997). Stimulation of rat soleus muscle for 10 s at 60 Hz increased

$[Na^+]_i$ by 58%, which was associated with an increase in NKA activity by ~15-fold (Everts and Clausen 1994).

2.7 Inactivity, immobilisation and exercise training effects on skeletal muscle NKA

2.7.1 Inactivity and immobilisation effects on skeletal muscle NKA

Numerous studies have indirectly investigated the effects of inactivity on muscle NKA content in humans, by measuring the impacts of injury. In participants with shoulder impingement syndrome, the NKA content in deltoid muscle decreased by 26% compared to the non-injured shoulder (Leivseth and Reikerås 1994). In patients after complete spinal cord injury, the NKA content in vastus lateralis muscle was decreased by ~34% compared to the deltoid muscle (Ditor et al. 2004). In chronic spinal cord injury patients, 75%, 52% and 38% decreases in NKA α_1, α_2 and β_1 isoform abundances were reported compared to the control group, respectively (Boon et al. 2012). In healthy young adults with a torn anterior cruciate ligament, the vastus lateralis muscle NKA content was 20 % less and the α_2 isoform abundance was 63% lower in the knee-injured leg than the non-injured leg (Perry et al. 2015). The above findings from human injury studies, strongly suggest a reduction in NKA content occurs with muscle inactivity, but might also be due to the injury or iatrogenic effects (Madsen et al. 1993).

The vastus lateralis muscle [^{3}H]ouabain-binding site content and the NKA isoform abundances in mixed muscle fibre homogenates were unchanged after 23 day of unilateral lower limb suspension (ULLS); however, in single muscle fibres, the NKA α_3 isoform abundance decreased by 66% in type I, and NKA β_1 isoform abundance decreased by 40% in type II after ULLS (Perry et al. 2016). Several studies in animals have, however, shown that inactivity caused by immobilisation decreases skeletal muscle [^{3}H]ouabain binding site content. One week of hindlimb immobilisation reduced the [^{3}H]ouabain binding site

content in rat soleus muscle by 20 - 22% (Kjeldsen et al. 1986b) and by 25% (Zemkova et al. 1990). In guinea pigs, hindlimb immobilisation for 3 and 4 weeks decreased the [^{3}H]ouabain binding site content in the gastrocnemius muscles by 25% and 23%, respectively (Leivseth et al. 1992). In female sheep, nine weeks of hindlimb immobilisation reduced the [^{3}H]ouabain binding site content in the vastus lateralis muscle by 39% (Jebens et al. 1995). Following immobilisation, the [^{3}H]ouabain binding site content returned to baseline levels after only 7 days (Zemkova et al. 1990) and after 4 - 5 weeks of recovery (Leivseth et al. 1992). Table 2.3 presents the effects of hindlimb immobilisation on [^{3}H]ouabain binding site content in animal skeletal muscle.

Recent studies have found that short-term hindlimb suspension in the rat for only 6 - 12 hours and for 1 - 3 days, decreased the electrogenic transport activity of the NKA α_2 by ~96% and ~71%, respectively, but not the NKA α_1 isoform, as measured by the ouabain-sensitive changes in resting membrane potential (Kravtsova et al. 2016; Kravtsova et al. 2015). Neither study investigated the α_3 or β_1 - β_3 isoforms. Nothing is known about the effects of hindlimb immobilisation on NKA α_3 or on β_1 - β_3 isoform abundances, and the recovery of NKA isoform abundance following hindlimb immobilisation has also not previously been investigated. Therefore, the first study in this thesis investigated the effects of hindlimb immobilisation in rats on the soleus muscle [^{3}H]ouabain binding site content and the abundance of each of the NKA α_1 - α_3 and β_1 - β_3 isoforms, as well as the recovery of NKA isoform abundances following hindlimb immobilisation.

Table 2.3 Effects of hindlimb immobilisation on [^{3}H]ouabain binding site content in animal skeletal muscle.

Ref	Species	Age (weeks)	Number	Duration (weeks)	Muscle Measured	[^{3}H]ouabain binding site content (% Δ changes)	Time to Recover
1	Rat	12	5	1	Sol	↓ 20 - 22%	
2	Rat	8-10	NR	1	Sol	↓ 25%	3 days
3	Sheep	NR	NR	9	VL	↓ 39%	
4	Pigs	NR	42	3 - 4	WG	↓ 23 - 25%	4 weeks

Reference: 1, Kjeldsen et al. 1986; 2, Zemkova et al. 1990; 3, Jebens et al. 1995; 4, Leivseth et al. 1992. Symbols: (↓) decrease; (↔) no change; (-) not measured; (Δ) changes and (NR) not reported. Muscles: SOL, Soleus; WG, White Gastrocnemius; VL, Vastus Lateralis.

2.7.2 Effects of exercise training on skeletal muscle NKA content and isoform abundances

Numerous studies have investigated the effects of exercise training on NKA content and isoform abundances in human skeletal muscle. It is difficult to compare directly between these studies because of variations in the type and duration of training undertaken, the training status of the participants, as well as the actual measurements performed. For simplicity these training studies are grouped under four broad categories: sprint, resistance, submaximal intensity and combined types of exercise training. Differences in the actual training undertaken are indicated in each section.

2.7.2.1 Effects of sprint training

Sprint training on a cycle ergometer comprising repeated maximal intensity 30 s bouts over 7 weeks in untrained participants caused an increase in muscle NKA content of 16% (McKenna et al. 1993) and 8% (Harmer et al. 2006). However, adaptions in specific isoforms were not investigated in either study, and whether the increased NKA content in these studies with sprint training was because of upregulation in one or both of the key α_1 and α_2 isoforms are unclear (Table 2.4).

Two studies investigated the effects of 30 s sprint training on NKA isoform abundances, but neither measured NKA content. In healthy untrained participants, sprint training comprising repeated 30 s runs at 130% VO_{2max} over 7 weeks elevated NKA α_2 by 68% and β_1 (percentage not reported) isoform abundances, but the α_1 isoform abundance was not changed (Mohr et al. 2007). In trained endurance runners, changing the regular endurance training to sprint training comprising repeated 30 s running bouts at 90 - 95% maximal speed over 3 - 4 weeks increased the NKA α_1 isoform abundance by 29%, whereas no changes were observed in α_2 and β_1 isoform abundances (Iaia et al. 2008) (Table 2.4).

Two studies have investigated the effects of sprint training comprising repeated bouts of only very short duration on skeletal muscle NKA isoform. One study reported that sprint training comprising repeated 4 s bouts over 7 weeks, increased NKA β_1 isoform in type IIa fibres by 42%, with no training effects found for other (α_1, α_2, α_3, β_2 and β_3) NKA isoforms (Wyckelsma et al. 2015). Sprint exercise comprising repeated 6 s at 95% maximal speed over 12 weeks elevated NKA β_1 (percentage not reported), but the α_1 and α_2 isoform abundances were not changed with training (Mohr et al. 2007) (Table 2.4). Therefore, the effects of repeated 30 s bouts of sprint training on skeletal muscle NKA isoform adaptations, and which NKA α isoforms abundance were associated with the upregulation of skeletal muscle NKA content are not clear. Given different "housekeeping" and "contractile" roles for α_1 and α_2 respectively (Crambert et al. 2000; DiFranco et al. 2015; Clausen et al 2013), this gives important clues to the functional of upregulation of NKA content in skeletal muscle. Further, no studies have investigated the effects of 30 s sprint exercise training on skeletal muscle NKA α_3, β_2, and β_3 isoform abundances, which is important to further understand the NKA adaptability after sprint training. Thus, study two in this thesis investigated the effects of repeated bouts of 30 s maximal sprint training on skeletal muscle NKA content and on the abundance of each of the NKA α_1 - α_3, β_1 - β_3 isoforms in healthy young adults.

2.7.2.2 Effects of resistance training

In a cross-sectional comparison, older participants (age 68 years) with a 12 - 17 year history of strength training, had a muscle NKA content greater by 40% than untrained, age-matched subjects (Klitgaard and Clausen 1989). Two studies have investigated the effects of longitudinal resistance training on muscle NKA content. In healthy untrained participants, the muscle NKA content was increased by 16% following 12 weeks of resistance training (Green et al. 1999b). In well-trained athletes, varying the frequency of

training sessions from 1, 2 and 3 sessions per week for 3 months of resistance training did not increase the NKA content (Medbø et al. 2001). However, when results were pooled from these groups the NKA content increased by 15%, (Medbø et al. 2001), nevertheless noting that this significant increase was based on participants who had not equally trained. However, none of these studies investigated the effects of resistance training on the muscle NKA isoforms.

A third study did investigate the adaption of muscle NKA isoforms to resistance training, finding that six weeks of resistance training increased muscle NKA α_1, α_2 and β_1 isoforms by 37%, 21%, 33%, respectively, in control participants (Dela et al. 2004) (Table 2.4). However, the NKA content was not investigated, so it is unclear what the increase in functional NKA was.

Thus the effects of resistance training on skeletal muscle NKA content and isoform adaptions are incompletely understood; with the other three NKA isoforms α_3, β_2 and β_3 not studied. Therefore, study three in this thesis investigated the effects of resistance training in healthy young adults on skeletal muscle NKA adaptation, measuring NKA content and isoform abundances to ascertain which isoforms were upregulated to account for the likely increase in NKA content.

2.7.2.3 Effects of submaximal intensity training

Short-term training at submaximal exercise intensity and long-term training were effective at increasing NKA content and isoform abundances in healthy untrained participants. Six days of submaximal cycling for 2 hours/day at 60% VO_{2peak} increased the muscle NKA content by 13.6% (Green et al. 1993). After only three days of short-term submaximal cycling training for 2 hours/day at 60 - 65% VO_{2peak}, the muscle NKA content was increased by 9% (Green et al. 2004). Similarly, three days of prolonged submaximal cycling training for 2 hours/day at 60% VO_{2peak} increased the muscle NKA

content by 12% and NKA isoform α_1, α_2, α_2, β_1, β_1 and β_1 isoforms by 46, 42, 31, 19, 28, and 18%, respectively (Green et al. 2008). Ten days of endurance cycle training at 75% Vo_{2peak} increased the NKA α_1, α_2, and β_1 isoforms by 113%, 49% and 27%, respectively (Benziane et al. 2011). Endurance cycling training at moderate intensity of 68% VO_{2max} elevated muscle NKA content by 22% (Green et al. 1999a).

In moderately endurance-trained athletes, high-intensity running training at 93% HR_{max} over six weeks increased muscle NKA content by 15% (Madsen et al. 1994). In cross-country skiers, moderate intensity at (60 -70% VO_2max) and high intensity at (80 - 90% VO_2max) training over five months increased muscle NKA content by 16% (Evertsen et al. 1997).

Alternately, in well-trained male cyclist the muscle NKA content and NKA isoforms were not changed after seven sessions of 8 × 5 min intermittent cycling training at 80% peak power output (Aughey et al. 2007). Similarly, in healthy participants, muscle NKA content did not increase after 10 weeks of military training, however no details were provided about the types or intensity of the training (Kjeldsen et al. 1990) (Table 2.4). Thus even submaximal exercise intensity conducted for a prolonged period results in upregulation of muscle NKA if participants were not already highly trained and typically also.in well trained participants. This is likely due to increases in each of the key NKA isoforms.

2.7.2.4 Effects of combined different type of exercise training

Several studies have investigated the effects of a combination of different types of training on muscle NKA, but these showed inconsistent results; furthermore none of these studies also measured NKA content. In elite soccer players, combining 25 - 30 s all-out running bouts with aerobic high intensity training (small-sided soccer drills) elevated the muscle NKA α_2 isoform by 15%, with no changes in the α_1 and β_1 isoform abundances

(Thomassen et al. 2010). Combining 30 s running bout with high and moderate intensity aerobic training for 6 - 9 weeks did not increase the NKA α_1, but increased NKA α_2 by 68% and β_1 isoform abundances (percentage not reported) in trained endurance runners (Bangsbo et al. 2009). Adding 30 s run sprint training at 90% - 95% of maximal intensity for trained soccer players to their normal training programme, decreased NKA β_1 isoform by 13%, but did not elevate the α_1 or α_2 isoform abundances (Gunnarsson et al. 2012). Combining 30 s running bout with moderate intensity aerobic training for 40 - 80 days increased the NKA β_1 isoform by 39 - 58%, but did not change the α_1 or α_2 isoforms (Skovgaard et al. 2016). Finally, combining strength, speed endurance and aerobic training for 8 weeks increased the NKA β_1 isoform by 15%, but did not change the α_1 and α_2 isoforms (Vorup et al. 2016) (Table 2.4).

In summary, the muscle NKA content was upregulated with exercise training in almost all of those studies that measured it, even those with short duration training lasting 3 - 6 days. In thirteen studies that investigated the effects of training on muscle NKA content, an increase in NKA content was reported in eleven of these studies (McKenna et al. 1993; Harmer et al. 2006; Green et al. 1999a; Medbo et al. 2001; Wyckelsma 2017; Evertsen et al., 1997; Madsen et al. 1994; Edge et al. 2013; Green et al., 2004; Green et al., 1993; Green et al. 2008), being unchanged in two studies (Kjeldsen et al. 1990; Aughey et al. 2007).

However, in contrast, the NKA isoform abundances, particularly the α_1 and α_2, showed marked variations in response to exercise training. This may be due to different impacts of the type and intensity of the training, of recovery period, and or of the training status of the participants. Another possible explanation for inconsistencies in the responses of the NKA isoform abundances is variability and methodological approaches using the Western blot assay. Sixteen studies have investigated the responses of NKA isoforms to

exercise training. For the α_1 isoform, increases were reported in six studies (Iaia et al. 2008; Dela et al. 2004; Nielsen et al. 2004; Benziane et al., 2011; Green et al. 2004; Green et al. 2008, and with α_1 unchanged in eight studies, Mohr et al. 2007; Wyckelsma et., 2015; Aughey et al. 2007, Bangsbo et al., 2009; Thomassen et al., 2010; Gunnarsson et al., 2012, Vorup et al. 2016; Skovgaard et al. 2016). Increases in α_2 isoform were reported in seven studies (Mohr et al. 2007; Dela et al. 2004; Nielsen et al. 2004; Benziane et al. 2011; Green et al. 2008; Bangsbo et al. 2009; Thomassen et al. 2010), being unchanged in nine studies (Iaia et al. 2008; Mohr et al. 2007;Wyckelsma et. 2017; Aughey et al. 2007; Green et al. 2004; Gunnarsson et al. 2012; Wyckelsma et. 2015; Vorup et al. 2016: Skovgaard et al. 2016). The α_3 isoform was measured in five studies, being increased in two (Wyckelsma et al. 2015; Green et al. 2008), but unchanged in three others (Wyckelsma 2017; Aughey et al. 2007; Benziane et al. 2011). The β_1 isoform was measured in sixteen studies, being reported to increase in seven (Dela et al., 2004; Mohr et al. 2007; Benziane et al., 2011; Green et al. 2008; Gunnarsson et al. 2012; Vorup et al. 2016: Skovgaard et al. 2016), being unchanged in eight studies (Iaia et al. 2008; Wyckelsma et. 2015; Wyckelsma 2017; Aughey et al., 2007; Nielsen et al. 2004; Green et al. 2004; Bangsbo et al. 2009; Thomassen et al 2010). The β_2 isoform was measured in five studies, being unchanged in four studies (Wyckelsma et. 2015; Wyckelsma et. 2017; Aughey et al., 2007; Benziane et al. 2011) and increased in one study (Green et al. 2008), while the β_3 isoform was measured in four studies, being reported increased in one study (Green et al., 2008), being unchanged in three studies (Wyckelsma 2017; Aughey et al. 2007; Wyckelsma 2017).

It has been assumed from rodent studies that an increase in [^{3}H]ouabain binding site content, reflecting an increase in the α_2 isoform, would also be the case in human skeletal muscle, but there is a lack of evidence to support this. Increased NKA content

accompanied by an upregulation of α_2 isoform was only reported in one study (Green et al. 2008), being unchanged in one other (Aughey et al. 2007). The increased NKA content associated with upregulation in the α_1 isoform was reported in three studies (Green et al. 2004; Green et al. 2008; Wyckelsma et al. 2017), being unchanged in one study (Aughey et al. 2007). Thus it cannot be concluded whether the increased NKA content with training was related to an upregulation of which of the key α_1 and α_2 isoform abundances. Therefore, studies two and three in this thesis address this issue investigating the upregulation of both skeletal muscle NKA content and NKA isoform protein abundance after sprint and resistance exercise training.

Table 2.4 The effects of exercise training on changes in skeletal muscle NKA in healthy humans

Ref	Number Male/Female	Age (yr) mean	Participant status	Training duration (weeks)	Training Type	[^{3}H]ouabain binding site content (%Δ)	Isoform protein abundances (%Δ)					
							α_1	α_2	α_3	β_1	β_2	β_3
Sprint Training												
1	6 M	18	Healthy untrained	7	Repeated maximal cycle 4 - 10 x 30s sprint bouts x3 p/wk	↑16%	-	-	-	-	-	-
2	7 M/F	24	Healthy untrained (control group)	7	Repeated maximal cycle 4 - 10 x 30s sprint bouts x3 p/wk	↑ 8%	-	-	-	-	-	-
3	7 M	24	Healthy untrained active	7	Repeated 8 x 30s runs at 130%VO_{2max} x3 - 4 p/wk	-	↔	↑ 68%	-	↑ % NR	-	-
4	7 M	33	Moderately trained endurance runners	3 - 4	Repeated 8 - 12 x 30s running bouts at 90-95% x3 - 5 p/wk	-	↑ 29%	↔	-	↔	-	-
5	7 M	24	Healthy untrained active	7	Repeated 15 x 6s running at 95% maximal speed x3 - 5 p/wk	-	↔	↔	-	↑ % NR	-	-

Table 2.4 continued

Ref	Number Male/Female	Age (yr) mean	Participants status	Training duration (weeks)	Training Type	[^{3}H]ouabain binding site content (%Δ)	Isoform protein abundances (%Δ)					
							α_1	α_2	α_3	β_1	β_2	β_3
6	17 M	26	Healthy untrained active	4	Repeated 5 x 4s all-out sprint x3 p/wk	-	measured in single fibre					
							↔	↔	↑ 42% type II fibre	↔	↔	↔
Resistance training												
7	6 Sex NR	20	Healthy untrained not active	12	Resistance training 3 sets 6 - 8 RM x3 p/wk	↑16%	-	-	-	-	-	-
8	23 M	27	Well-trained athletes	7	Resistance training 5 sets 4 reps at 70% 1RM x1 - 3 p/wk load ↑ 2.5% p/wk	↑ 15%	-	-	-	-	-	-
9	6 M	25	Untrained not active (control group)	6	Resistance training wk 1 - 2 3 sets 10 reps at 50% 1RM wk 3 - 6 at 70 - 80% 1RM x3 p/wk	-	↑ 37%	↑ 21%	-	↑33%	-	-

Table 2.4 continued

Ref	Number Male/Female	Age (yr) mean	Participants status	Training duration (weeks)	Training Type	[^{3}H]ouabain binding site content (%Δ)	Isoform protein abundances (%Δ)					
							α_1	α_2	α_3	β_1	β_2	β_3
Submaximal and intensity training												
10	15 (9 M/6 F)	69	Untrained periodically active	12	High Intensity cycling x3 p/wk 4 x 4 min at 90-95% HR_{peak}	↑ 11%	measured in single fibre					
							↑ 30% in type II fibre	↔	↔	↔	↔	↔
11	20 (11 M/ 9 F)	18	Cross-country skiers trained	5 months	Skiing, moderate-intensity at 60 -70% VO_{2max} x7 p/wk for 40 min - 3 h run high-intensity at 80 - 90% VO_{2max} x2-3 p/wk for 10 min - 2 h or 40 s - 7 min	↑ 16%	-	-	-	-	-	-
12	39 M	30	Moderately endurance trained	6	High-intensity running at 93% HR_{max} x3 p/wk for 25 min	↑ 15%	-	-	-	-	-	-
13	12 F	19	Trained intermittent sport club level	5	High-intensity cycling x3 p/wk 6 - 10 x 2 min at 140% LT_{Dmax} load ↑ 10% p/wk	↑ 22- 26%	-	-	-	-	-	-
14	12 M	31	Trained cyclists/athletes	4	High-intensity cycling at 80% peak power output 8 x 5 min/7 session wk 1 x3/wk wk 2 - 3 x2/wk	↔	↔	↔	↔	↔	↔	↔

Table 2.4 continued

Ref	Number Male/Female	Age (yr) mean	Participants status	Training duration (weeks)	Training Type	[^{3}H]ouabain binding site content (%Δ)	Isoform protein abundances (%Δ)					
							α_1	α_2	α_3	β_1	β_2	β_3
15	6 M	25	untrained active	7	High intensity knee extensor 15 intervals at ~150% VO_{2max} x3 - 5 p/wk	-	↑ 29%	↑15%	-	↔	-	-
16	9 M	23	Untrained not active	10 days	Endurance cycling 4 d for 40 min at 75% VO_{2max} 1 d for 60 min at 75% VO_{2max} 1 d for 90 min at 75% VO_{2max} 4 d for 6 x 5 min at 90 - 100% VO_{2max}	-	↑130%	↑ 49%	↔	↑ 27%	↔	-
17	7 Sex NR	21	Healthy untrained not active	11	Endurance cycling 2 h/ d at 68% VO_{2peak} x5 - 6 p/wk	↑ 22%	-	-	-	-	-	-
18	7 M	20	untrained not active	6 days	Cycling for 2 h/ d at 60-65% VO_{2peak}	↑ 9%	↑ 19%	↔	-	↔	-	-
19	9 M	19	Untrained periodically active	6 days	Cycling for 2 h/ d at 65% VO_{2max}	↑ 13%	-	-	-	-	-	-
20	12 (6 M/6 F)	19	untrained active	3 days	Short term model cycling for 2 h/ d at ~60% VO_{2peak}	↑ 12%	↑ 46%	↑ 42%	↑31%	↑ 19%	↑ 28%	↑ 18%

Table 2.4 continued

Ref	Number Male/Female	Age (yr) mean	Participants status	Training duration (weeks)	Training Type	[^{3}H]ouabain binding site content (%Δ)	Isoform protein abundances (%Δ)					
							α_1	α_2	α_3	β_1	β_2	β_3
Combined training												
21	17 M	34	Moderately trained endurance runners	6 - 9	Combined repeated 8 - 12 x 30s running bouts at 95% max speed with high at >85 HR_{max} and low intensity at < 75 HR_{max} aerobic training 4 x 4 min x2 - 3 p/wk	-	↔	↑ 68%	-	↔	-	-
22	18 M	23	Elite trained soccer players	2	Combined repeated 10 - 12 x 25 - 30s running bouts aerobic high intensity (small sided soccer drills) 10 training sessions	-	↔	↑ 15%	-	↔	-	-
23	18 M	23	Sub-elite trained soccer players	5	Adding 6 - 9 x 30s at 90% -95% max intensity to normal training program x1 p/wk	-	↔	↔	-	↓13%	-	-
24	16 M	38	trained endurance runners	8	Compound training strength training 1 - 4 x 4 -10 rep aerobic training 2 - 17 km running at ~80% HR_{max} 8 x 2 min ruining buts at >90 HR_{max} repeated 4 - 8 x 30s	-	↔	↔	-	↑ 27%	-	-

Table 2.4 continued

Ref	Number Male/Female	Age (yr) mean	Participants status	Training duration (weeks)	Training Type	[^{3}H]ouabain binding site content (%Δ)	Isoform protein abundances (%Δ)					
							α_1	α_2	α_3	β_1	β_2	β_3
25	18 (14 M/4 F)	28	Moderately trained runners	40 - 80 days	Combined repeated 8 - 12 x 30s running bouts all-out Moderate intensity aerobic training at 30 - 60% HR_{max} for 30 - 60 min x2/wk	-	↔	↔	-	↑ 39 - 58%	-	-

References: 1, McKenna et al. 1993; 2, Harmer et al. 2006; 3, Mohr et al. 2007; 4, Iaia et al. 2008; 5, Mohr et al. 2007, 6, Wyckelsma et. 2015, 7, Green et al. 1999; 8, Medbo et al. 2001; 9, Dela et al. 2004; 10 ,Wyckelsma 2017; 11, Evertsen et al. 1997; 12, Madsen et al. 1994; 13, (Edge et al. 2013); 14, Aughey et al. 2007; 15, (Nielsen et al. 2004a); 16, Benziane et al. 2011; 17, Green et al. 1999; 18, Green et al. 2004; 19, Green et al. 1993; 20, Green et al. 2008; 21, Bangsbo et al. 2009; 22, Thomassen et al. 2010; 23, Gunnarsson et al., 2012; 24, Vorup et al. 2016; Skovgaard et al. 2016. **Symbols:** (↑) P<0.05 increased; P<0.05 (↓) decreased; (↔) No effects; (-) not measured, **(Δ)** changes; (1RM) 1 repetition maximum; (HR) heart rate, (VO_2) rate of oxygen consumption, (LT_{Dmax}) lactate threshold, (p/wk) per week and (NR) not reported.

2.8 Chronic kidney disease (CKD) and skeletal muscle NKA

2.8.1 Effects of CKD on muscle function

CKD is associated with protein energy wasting, which is a term to describe loss of body protein and fuel reserves (Fouque et al. 2008; Carrero et al. 2008). This includes a reduction in muscle mass (Fouque et al. 2008) and impaired mitochondrial function (Lim et al. 2000; Lim et al. 2002; Gamboa et al. 2016), which result in a limited physical function (Rhee and Kalantar-Zadeh 2014; Painter and Roshanravan 2013). This is clearly seen in reduced muscle strength and exercise aerobic power as measured by VO_{2peak} and greater fatigability. Numerous studies have reported 30 - 70% reduction in VO_{2peak} in CKD compared to healthy controls (Hsieh et al. 2006; Painter et al. 2002; Sangkabutra et al. 2003; Van Den Ham et al. 2005; Petersen et al. 2011). Muscle strength was reduced by 20 - 60% in CKD as measured by 1RM, isokinetic peak torque or knee extensor (DePaul et al. 2002; Van Den Ham et al. 2005; Fahal et al. 1997; Petersen et al. 2009). During isometric dorsiflexion contractions time to fatigue was decreased by ~30% (Kemp et al. 2004). Muscle fatigability during isometric dorsiflexion contractions was 3-fold greater (Johansen et al. 2005) and ~ 1.6-fold greater during isokinetic contraction in CKD compared than controls (Petersen et al. 2011).

2.8.2 Effects of CKD on muscle NKA and isoform abundances

It has been suggested that impaired K^+ regulation may contribute to reduced exercise performance and greater muscle fatigue in CKD (Sangkabutra et al. 2003; Petersen et al. 2009). During incremental exercise plasma [K^+] was higher than control at rest, during and after exercise (Petersen et al. 2009). The rate of K^+ release from contracting muscle is dependent on the balance of K^+ efflux and K^+ reuptake and is regulated by the NKA in skeletal muscle (Nielsen and Clausen 2000). Thus any depression in muscle NKA may

also enhance fatigue and reduce exercise performance (Clausen 2008). One study reported that depressed NKA activity contributes to poor exercise performance and muscle fatigability in CKD patients (Petersen et al. 2011). CKD is associated with low levels of physical activity and reduced muscle function (Avesani et al. 2011), given that physical inactivity have shown to impaired muscle NKA (section 2.7). Thus there is a possibility that any reduced in NKA may be result of inactivity and not related to the disease itself. However, there no studies have investigated the effect of inactivity on NKA in patients with CKD. Hence, further research is needed to determine the effects of inactivity on muscle NKA in patients with CKD.

Skeletal muscle contains the large pool of NKA in the body (Clausen 2010). Hence, there is considerable interest in whether CKD depressed muscle NKA content and NKA isoform abundances. However, one study reported no difference in skeletal muscle NKA content and isoform abundances in CKD patients, including haemodialysis patients and renal transplant recipients, compared to controls (Petersen et al. 2011). In rats with CKD, there was no difference in the muscle [^{3}H]ouabain binding site content in the epitrochlearis muscle compared to control (Druml et al. 1988). Given impaired K^+ regulation during exercise, findings of a lack of effect of CKD on skeletal muscle NKA and isoform abundances are surprising and deserving of further investigation. Therefore, study four investigated whether NKA content and NKA isoform abundances were reduced in patients with CKD compared to healthy controls.

2.8.3 Effects of exercise training on skeletal muscle NKA and isoform abundances in CKD.

Patients with CKD have been shown to adapt to exercise training. In patients with CKD, a training program comprising swimming, ball games and resistance training for 6 months increased muscle mean cross-section area, type I and type II muscle fibre area by 26, 29 and 24%, respectively; VO_{2peak} was also increased by 48% (Kouidi et al. 1998). In patients with CKD, resistance training for 12 weeks increased muscle mitochondrial content by 12% and type I muscle fibre area by17% (Balakrishnan et al. 2010) and muscle strength by 13 - 26% (Johansen et al. 2006; Headley et al. 2002). Endurance cycling training over 12 weeks improved VO_{2peak} by 22 % and muscle strength by 16% in patients with CKD (Storer et al. 2005).

Given the effects of exercise training in upregulation of NKA content and isoform abundances (section 2.6), which is important for skeletal muscle function (Clausen 2013b), it is important to also understand the impact of training on skeletal muscle NKA in patients with CKD. Whilst in healthy participants, exercise training increased NKA content and NKA isoform abundances (Table 2.4), no study has however, investigated the effects of exercise training on muscle NKA content and isoform abundances in patients with CKD. This is important to determine as it remains unknown whether skeletal muscle in CKD patients undergoes similar adaptations in NKA as found in healthy individuals. There is growing interest in the clinical application of high intensity interval training, as this may be a more enticing option to encourage CKD patients to participate in regular exercise. It was therefore important to evaluate the effects of both types of training on the adaptability of muscle NKA in patients with CKD. Therefore study four investigated the effects of moderate intensity continuous (MIIT) and high intensity

interval training (HIIT) on muscle NKA content and on the abundance of each of α_1 - α_3, β_1 - β_3 isoforms in patients with CKD.

2. 9 Aims and hypotheses

2. 9.1 Study One

Study one investigated the effects of hindlimb immobilisation and testosterone suppression via castration surgery in rats on the [^{3}H]ouabain binding site content and NKA isoform abundances in soleus muscle.

Hypothesis: The skeletal muscle [^{3}H]ouabain binding site content and NKA α_2 and β_1 isoform abundances will be reduced with both hindlimb immobilisation and with testosterone suppression.

2.9.2 Study Two

Study two investigated which NKA α and β isoforms were associated with an anticipated upregulation of skeletal muscle NKA content following 7 weeks of sprint exercise training in healthy young adults.

Hypothesis: Sprint training in healthy young adults would increase skeletal muscle NKA content, which would be associated with upregulation of the each of the NKA α_1, α_2, and β_1 isoforms.

2.9.3 Study Three

Study three investigated which NKA α and β isoform abundances were associated with an anticipated upregulation of skeletal muscle NKA content following 7 weeks of resistance exercise training in healthy young adults.

Hypothesis: Resistance training in healthy young adults would increase skeletal muscle NKA content, which would be associated with upregulation of the each of the NKA α_1, α_2, and β_1 isoforms.

2.9.4 Study Four

Study four investigated first, if any differences existed between patients with CKD and healthy participants in skeletal muscle NKA content and isoform abundances, and second, whether moderate or high intensity exercise training for 12 weeks in patients with CKD would increase skeletal muscle NKA content and isoform abundances.

Hypotheses: There would be no differences in skeletal muscle NKA content and isoform abundances between patients with CKD and healthy participants.

Moderate or high intensity training exercise would each increase skeletal muscle NKA content and this would be associated with upregulation of the each of the NKA α_1, α_2, and β_1 isoforms.

Chapter 3: Effects of hind limb immobilisation and castration on [^{3}H]ouabain binding site content and NKA isoform abundance in rat soleus muscle

3.1 Introduction.

Immobilisation causes substantial muscle atrophy, most likely due to both decreased protein synthesis and increased proteolysis and is well known for its detrimental effects on muscular function, including loss of muscle strength (Järvinen and Lehto 1993; Kujala et al. 1997; Powers et al. 2005). The effects of immobilisation on factors affecting muscle excitability are thus of interest. In skeletal muscle, the NKA plays a critical role in muscle function via maintenance of the [Na^+] and [K^+] gradients across sarcolemmal and t-tubular membranes, maintaining high intracellular [K^+] and a low intracellular [Na^+] (Clausen 2003a). Hence, any decreases in muscle NKA may adversely impact on the regulation of muscle [Na^+], [K^+] and excitability during exercise and fatigue (McKenna et al., 2008; McKenna et al., 2012; Perry et al. 2015). In skeletal muscle, the NKA comprises three α (α_1 - α_3), and three β (β_1 - β_3) isoforms (Murphy et al. 2004). Hindlimb immobilisation for one week decreased the [^{3}H]ouabain binding site content in rat soleus muscle by 20-30% (Kjeldsen 1986; Zemkova et al. 1990). Hindlimb immobilisation for 3 - 4 weeks reduced the [^{3}H]ouabain binding site content by 23 - 25% in guinea pig gastrocnemius muscle (Leivseth et al. 1992) and by 39% in sheep vastus lateralis muscle (Jebens et al. 1995). However, none of these studies investigated which specific NKA isoforms were affected by hindlimb immobilisation. Two recent studies reported that hindlimb immobilisation in rat soleus muscle for only six to twelve hours, and for one to three days decreased the electrogenic activity of the NKA α_2 isoform by 72% and 89%, respectively, but did not decrease the α_1 isoform electrogenic activity (Kravtsova et al. 2015; Kravtsova et al. 2016). Both studies reported that the α_1 isoform protein abundances

did not change, whilst in contrast the α_2 isoform abundances actually increased after hindlimb immobilisation, although this increase might be due to a transient stress response to hindlimb suspension (Kravtsova et al. 2015). Neither study investigated the [^{3}H]ouabain binding site content, or other NKA (α_3, and β_1 - β_3) isoforms. The [^{3}H]ouabain binding site content following hindlimb immobilisation returned to baseline levels after only 7 d (Zemkova et al. 1990) and after 4 - 5 weeks of recovery (Leivseth et al. 1992). However, recovery of NKA isoform abundances following hindlimb immobilisation has not previously been investigated and the effects of hindlimb immobilisation on skeletal muscle NKA α_3, β_1 - β_3 isoform abundances are unknown. Given the importance of the β isoform in regulation of NKA activity (Scheiner-Bobis 2002; Hundal et al. 1994) it is beneficial to also understand the impact of hindlimb immobilisation on β_1 - β_3 isoform abundances. Moreover, no study has investigated the effects of immobilisation using the combined measures of the [^{3}H]ouabain binding site content method and western blot measures of the α_2 isoform abundance; this is important for further understanding of the effects of hindlimb immobilisation and recovery in rat muscle.

Testosterone is a male sex hormone principally synthesised in the testes, with reduced testosterone leading to decreased muscle mass and strength (Leichtnam et al. 2006). Testosterone reduction can occur as a consequence of age (Nigro and Christ-Crain 2012), prostate cancer (Schatzl et al. 2001; Hoffman et al. 2000), and type 2 diabetes (Stellato et al. 2000; Traish et al. 2009). Importantly, no study has investigated the impacts of testosterone reduction on NKA in skeletal muscle, although there is evidence of modulation in other tissues. Testosterone reduction via castration led to 47 - 73% decreases in NKA activity in rat erythrocyte membranes, as measured over a 1 - 9 month

period compared to sham surgery (Sheng-qi et al. 1994). In rats, 4 weeks following castration NKA activity was decreased in the mediobasal hypothalamus; injection of 50 µg testosterone to castrated rats elevated NKA activity 4-fold in the preoptic-suprachiasmatic region of the brain, compared sham rats (Guerra et al. 1987). Given the α_3 isoform is highly abundant in the brain (Bøttger et al. 2011), this result most likely reflects α_3 isoform activity. Given the important of testosterone in muscle mass and strength (Kvorning et al. 2006; Herbst and Bhasin 2004; Sinha-Hikim et al. 2002), it is of interest to determine whether testosterone suppression would reduce the skeletal muscle [^{3}H]ouabain binding site content and NKA isoform abundances in skeletal muscle. Whether testosterone reduction and hindlimb immobilisation might have combined effects on skeletal muscle NKA content and NKA isoform abundances is also unknown. This study therefore investigated the effects of hindlimb immobilisation and testosterone suppression via castration in rats on the [^{3}H]ouabain binding site content and NKA isoform protein abundances in soleus muscle. It was hypothesised that the hindlimb immobilisation and castration would lead to reduced skeletal muscle [^{3}H]ouabain binding site as well as NKA α_2 and β_1 isoform abundances.

3.2 Methods and procedures

3.2.1 Study Design

This study was part of a larger scale study (Lin et al. 2016). Due to muscle limitation only soleus muscle was available to be measured here. F344 inbred male rats aged ~8 weeks underwent castration or sham surgery; each then recovered for 7 d whilst consuming a standard diet (Lin et al. 2016). Rats in both castration and sham groups were then divided into two sub-groups that underwent unilateral hindlimb immobilisation to induce substantial muscle atrophy, or served as non-immobilisation controls. The unilateral hindlimb immobilisation group had one hindlimb casted for 10 d, and a non-cast hindlimb (Figure 3.1). The sham group comprised non-immobilisation controls (n = 8), unilateral immobilisation (n = 8), and after 14 d of recovery (n = 8); the castration group comprised, non-immobilisation controls (n = 7), unilateral immobilisation (n = 8), and after 14 d of recovery (n = 8). The immobilisation and testosterone interventions and collection of all tissue samples were completed at the Western Centre for Health Research and Education at Sunshine Hospital, with all experiments and procedures approved by the Victoria University Animal Ethics Committee.

3.2.2 Castration and Sham Surgery Procedures

The testosterone concentration was manipulated via orchiectomy surgery, which led to a reduction in testosterone of more than 90% (Lin et al. 2016). The animals were anesthetised by 4% isoflurane anaesthesia in an induction chamber before being transfered to a face mask until the animals were unresponsive to tactile stimuli. Buprenorphine hydrochloride (0.5 mg/kg Meloxicam, Therapon, Burwood, VIC, Australia) was injected at least 30 minutes before induction, and then the flank was shaved on both side, area sterilised and incision made. A ligature was placed around the vas

deferens and the blood vessels and each testis removed. After surgery animal were allowed to recover with pain management (0.5mg/kg Meloxicam, Therapon, Burwood, VIC, Australia). In sham surgery conducted in control rats, the procedures were identical, except that the testes were exposed but not removed.

3.2.3 Immobilisation Procedure

A unilateral hindlimb immobilisation was performed in all rats except for the non-casted control animals. Following light anaesthesia with 2 - 4% isoflurance, the right limb of each animal was immobilised with casting material in a neutral foot position, such that neither the extensor digitorum longus (EDL) nor soleus muscles were fully lengthened or shortened, respectively. The hindlimb was wrapped with several layers of casting material before being immobilised by a thermoplastic splint. Additional tape was placed to secure the casting material in place. The contra-lateral leg remained loose to allow for ambulation and was utilised as an active control for the immobilised leg of each animal. The casts were inspected and repaired daily as necessary. At the end of the 10 d immobilisation, the same anaesthetic procedures were used to remove the casting material to begin the regrowth period.

3.2.4 Animal sacrifice and muscle sampling

The animals were deeply anaesthetised with sodium pentobarbital (60 mg/kg; Therapon, Burwood, VIC, Australia), and soleus muscles were obtained, before the immobilisation period from non-immobilised control only, following 10 days immobilisation and after 14 days of recovery from the cast and non-cast leg (Fig 3.1). The muscle samples were then snap-frozen in liquid nitrogen before being stored at -80 °C for later analyses. Immediately after excision the muscle animals were culled via an overdose of anaesthetic (sodium pentobarbital 375 mg/kg; Therapon, Burwood, VIC, Australia).

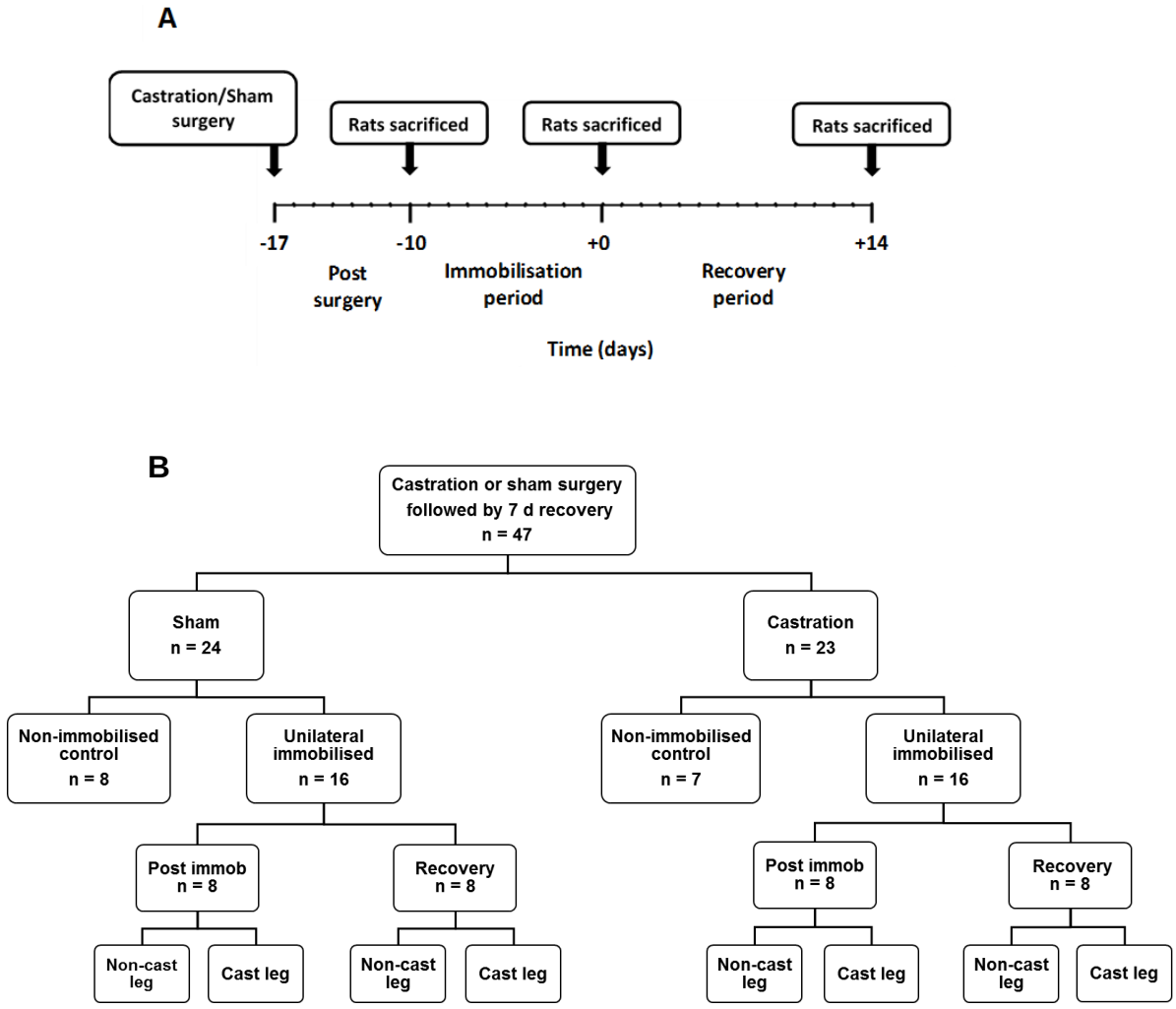

Figure 3.1. Experimental design overview showing A) time line of surgery, immobilisation and recovery, and B) groups subgroups and sample sizes.

Animals were sacrificed and soleus muscles were obtained from F344 inbred male rats at each of the following time points: 7 d after surgery and just prior to immobilisation period (-10 d) only from non-immobilised controls; immediately following the 10 d immobilisation period (+0); and after 14 d of recovery (+14) from both sham and castration groups.

3.2.5 [^{3}H]ouabain binding site content

Approximately 20 mg of muscle was analysed in quadruplicate using the vanadate-facilitated [^{3}H]ouabain binding content method as previously described (Nørgaard et al. 1984, Petersen et al. 2005). Each sample was washed for 2 x 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). Muscle samples were then incubated for 2 h at 37°C in vanadate buffer with the addition of [^{3}H]ouabain (2.0 Ci^{-1}ml and 10^{-6} M, PerkinElmer, Boston, MA). 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 blotted on filter paper and weighed before being soaked overnight in 500 µl of 5% trichloroacetic acid and 0.1 mM ouabain. Following this, 2.5 ml of scintillation cocktail (Opti-Fluor, Packard, PerkinElmer, Boston, MA) was added before liquid scintillation counting of [^{3}H]ouabain. The [^{3}H]ouabain binding site content was then calculated on the basis of the sample wet weight and specific activity of the incubation buffer and samples (Petersen et al. 2005; Nørgaard et al., 1984). The final [^{3}H]ouabain binding site content was calculated using a correction factor of 1.33 as previously described for rat muscle (Clausen et al. 2011) to allow for impurity of [^{3}H]ouabain, loss of specifically bound [^{3}H]ouabain during washout and incomplete saturation during the equilibration of muscle with [^{3}H]ouabain. The [^{3}H]ouabain binding site content was expressed as pmol.g ww^{-1}.

3.2.6 Western Blotting

To determine skeletal muscle Na^+,K^+-ATPase α and β isoform relative protein abundances, approximately 20 mg of frozen muscle was used and analysed using western blotting (Perry et al. 2013). Muscle proteins were lysed in ice-cold buffer containing 20 mM Tris pH 7.8 (Bio-Rad Laboratories, Hercules, CA), 137 mM NaCl, 2.7 mM KCl

(Merck, Kilsyth, Australia), 1 mM $MgCl_2$, 5 mM $Na_4O_7P_2$, 10 mM NaF, 1% Triton X-100, 10% Glycerol (Ajax Finechem, Australia), Protein Inhibitor Cocktail (P8340). All reagents were analytical grade (Sigma-Aldrich, St Louis, MI). Samples were homogenised (1:40 dilution) using a tissueLyser II (QIAGEN, Hilden, Germany) followed by gentle rocking for 60 min at 4°C. Protein concentration of the homogenate was determined using a commercially available kit (DC Protein Assay, Bio Rad Laboratories, USA).

Repeated steps of centrifugation of muscle and membrane separation have resulted in very low recovery of NKA, yielding a final sample that may be unrepresentative of the whole muscle NKA (Hansen & Clausen, 1988). Therefore, muscle Na^+,K^+-ATPase isoform analyses did not include any membrane isolation steps, to maximise recovery of Na^+,K^+-ATPase enzymes (Murphy et al. 2004). Aliquots of the muscle homogenate were mixed with Laemmli sample buffer and proteins were separated on a 26 well Criterion Stain Free precast gels (8 - 16%, Criterion TGX, Bio-Rad Laboratories, USA) for 45 min at 200 V and 400 mA.

For the analysis of protein abundance of the Na^+,K^+-ATPase isoforms (α_1, α_2, α_3, β_1, β_2 and β_3), 10 µg of total protein per sample were loaded in each gel. To ensure that blot density was within the linear range of detection (Murphy and Lamb 2013), a four to five-point (2.5 - 12.5 µg) calibration curve of whole-muscle crude homogenate was loaded onto every gel. The homogenate was prepared from an equal amount of 5 µg from each sample.

Following electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (TurboTransfer pack, Bio-Rad Laboratories, USA) for 7 min at 320 mA using

the semi-dry Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, USA). Membranes were blocked in PBST buffer (10 mM Tris, 100 mM NaCl, 0.02% Tween-20) containing 5% non-fat milk, for 1 h at room temperature. After being washed (4 x 8 min in TBST), membranes were incubated with the appropriate primary antibody overnight at 4°C. Primary antibodies were diluted in PBS buffer containing 0.1% NaN_3 and 0.1% albumin bovine serum. Following incubation with the primary antibodies, membranes were washed in PBST buffer (4 x 5 min) and incubated with the appropriate anti-rabbit (PerkinElmer # NEF812001EA) or anti-mouse (PerkinElmer # NEF822001EA) horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After washing the membranes in PBST (4 x5 min), membranes were incubated for 5 min with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo, Waltham, MA, USA), then stain free images were taken using a ChemiDoc Imaging system (Bio-Rad Laboratories, USA). The densities of samples were expressed relative to the total protein on the gel and then normalised to the calibration curve (Murphy and Lamb 2013).

The following antibodies were used for NKA isoform α_1 (monoclonal α6F, developed by D. Fambrough, obtained from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa, USA), α_2 (polyclonal anti-HERED, Millipore, # 07- 674), α_3 (monoclonal, Thermo Scientific, Rockford, IL, # MA3-915), β_1 (Millipore, # 05- 382), β_2 (Proteintech # 22338-1-AP), β_3 (BD Bioscience, # 610992) and β_3 (BD Bioscience, # 610993). Validation of antibodies was performed with positive and negative controls using mixed human skeletal muscle homogenate, rat EDL, rat soleus, rat heart and rat kidney as shown in Fig. 3.2.

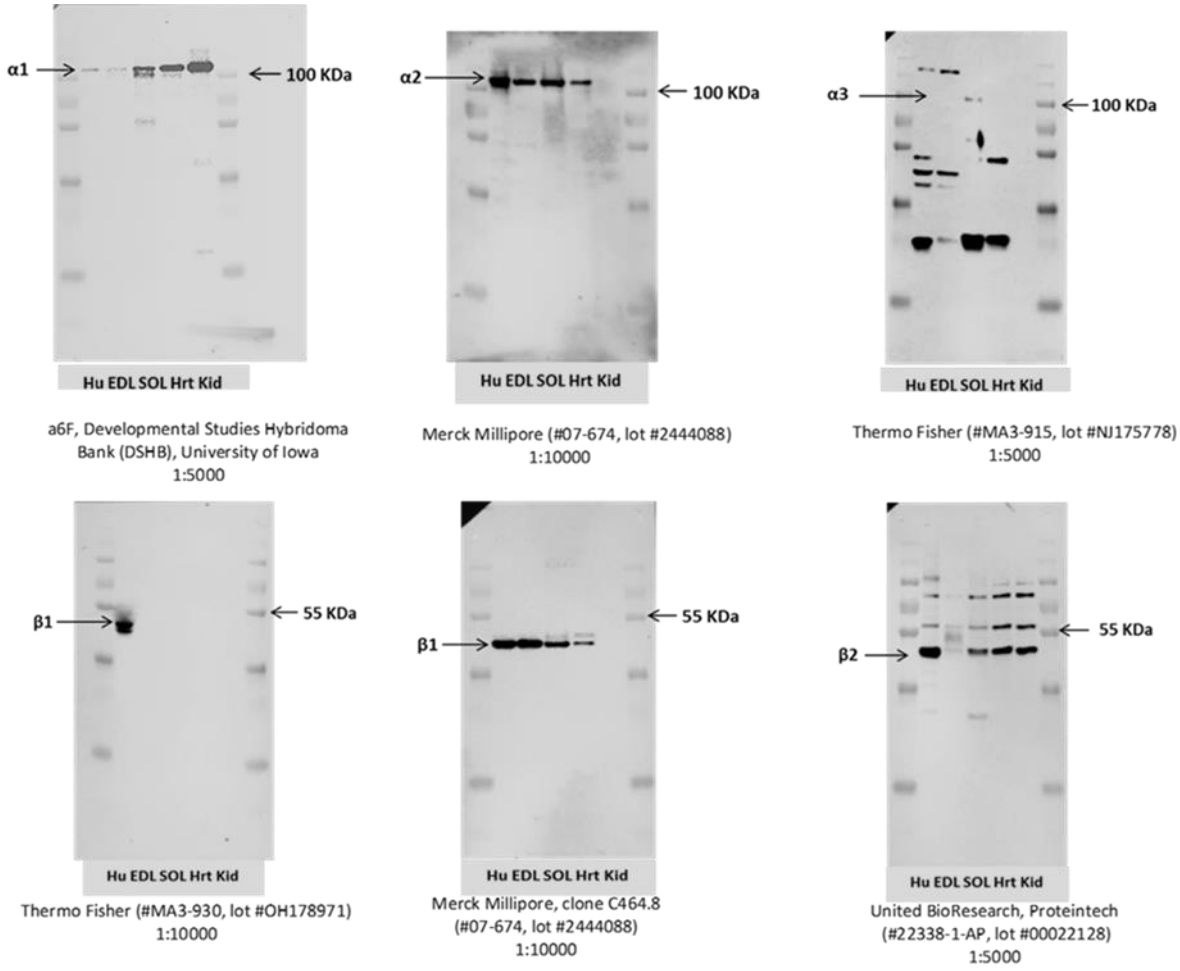

Figure 3.2. Validation of antibodies used to quantify NKA isoforms, showing the name of each antibody and the dilution factor.

Total of 10 µg protein of mixed human skeletal muscle homogenate (Hu), rat extensor digitorum longus muscle (EDL), rat soleus muscle (SOL), rat heart (Hrt) and rat kidney (Kid) were loaded onto each gel. Values at right indicate molecular weight of bands and at left indicate the NKA isoform.

The NKA α_1 antibody (#a6F) detected a band at the predicted molecular weight ~105 kDa in human skeletal muscle, rat EDL muscle, rat SOL muscle, rat heart and rat kidney. The NKA α_2 polyclonal antibody (Millipore #07-674) detected a clear band at the predicted molecular weight of ~105 kDa in human skeletal muscle, rat EDL muscle, rat SOL muscle and rat heart, but was absent in rat kidney. The NKA α_3 antibody (Thermo Scientific #MA3-915) detected band at the predicted molecular weight of ~105 kDa in rat SOL muscle but was absent in human skeletal muscle, rat EDL muscle, rat heart and rat kidney. The NKA β_1 antibody (Thermo Scientific #MA3-930) specifically designed to detect β_1

protein only in human muscle detected a clear band at the predicted molecular weight ~50kDa in human skeletal muscle and was absent in rat EDL muscle, rat SOL muscle, rat heart and rat kidney. The second NKA β_1 antibody (Millipore, # 05- 382) detected a band at the expected molecular weight of ~50kDa in human skeletal muscle, rat EDL muscle, rat SOL muscle and rat heart, but was absent in rat kidney. The NKA β_2 antibody (Proteintech #22338-1-AP) detected a band at the predicted molecular weight in human skeletal muscle, rat EDL muscle (albeit weak), rat SOL muscle, rat heart and rat kidney. The NKA β_3 could not be detected by two different antibodies used (BD Bioscience, # 610993) and BD Bioscience, # 610992).

3.2.7 Statistical Analysis

A one-way ANOVA was used to assess the effects of immobilisation and castration on soleus muscle [^{3}H]ouabain binding site content and NKA isoform abundances, within the respective sham group and castration groups. Pairwise comparisons were made using the Least Significant Difference (LSD) post-hoc test. To determine the effect of castration only on soleus muscle NKA [^{3}H]ouabain binding site content and NKA isoform abundances, the non-immobilised control in the castration group was compared to non-immobilised control in sham group, using an unpaired student t-test. Data were assessed for normality using the Shapiro-Wilk test and all data were normally distributed. Statistical analyses were conducted using SPSS version 24. Effect size was calculated using Cohen's *d*, where <0.2, 0.2-0.5 and 0.5-0.8 and >0.8 are considered trivial, small, moderate and large, respectively (Cohen 1988). Data are presented as mean ± standard deviation (SD). Statistical significance was accepted at $P < 0.05$. All figures were created using SigmaPlot 13 (Systat Software, Inc).

3.3 Results

3.3.1 [^{3}H]ouabain binding site content

3.3.1.1 Comparison of non-immobilised controls in castration vs the sham groups

To determine the effects of testosterone suppression per se on soleus muscle [^{3}H]ouabain binding site content and NKA isoform abundances i.e. independent of hindlimb immobilisation, the non-immobilised control in the castration group was compared to the non-immobilised control in the sham group. There was no difference in the muscle [^{3}H]ouabain binding site content between sham and castrated non-immobilised controls (459.2 ± 103.8 vs. 452.6 ± 55.4 pmol.g wet weight^{-1}, p = 0.884; d = 0.073).

3.3.1.2 Within-sham group comparisons

There were significant differences between groups within sham group comparisons (p = 0.012). Post-immobilisation, the muscle [^{3}H]ouabain binding site content in the cast leg was 26% lower than in the non-cast leg (p = 0.023; d = 1.34) and ~34% lower (p = 0.001; d = 1.69) than in the non-immobilised control group (Fig. 3.3A). At 14 d recovery, the muscle [^{3}H]ouabain binding site content had recovered, such that there were no differences in the cast leg compared to either the non-cast leg (p = 0.391; d = 0.540) or non-immobilised control group (p = 0.833; d = 0.093, Fig. 3.3A).

3.3.1.3 Within-castration comparisons

There were significant differences between groups within castration group comparisons (p =0.001). Post immobilisation, the muscle [^{3}H]ouabain binding site content in the cast leg did not differ significantly from the non-cast leg (p = 0.163; d = 0.996), but was 34% lower than in the non-immobilised control group (p= 0.001; d = 3.03, Fig. 3.3B). At 14 d recovery, the [^{3}H]ouabain binding site content in the cast leg remained depressed, being

~34% lower than in the non-immobilised control group (p = 0.001; d = 2.02, Fig 3.3B), but was not different from the non-cast leg (p = 0.456; d = 0.30).

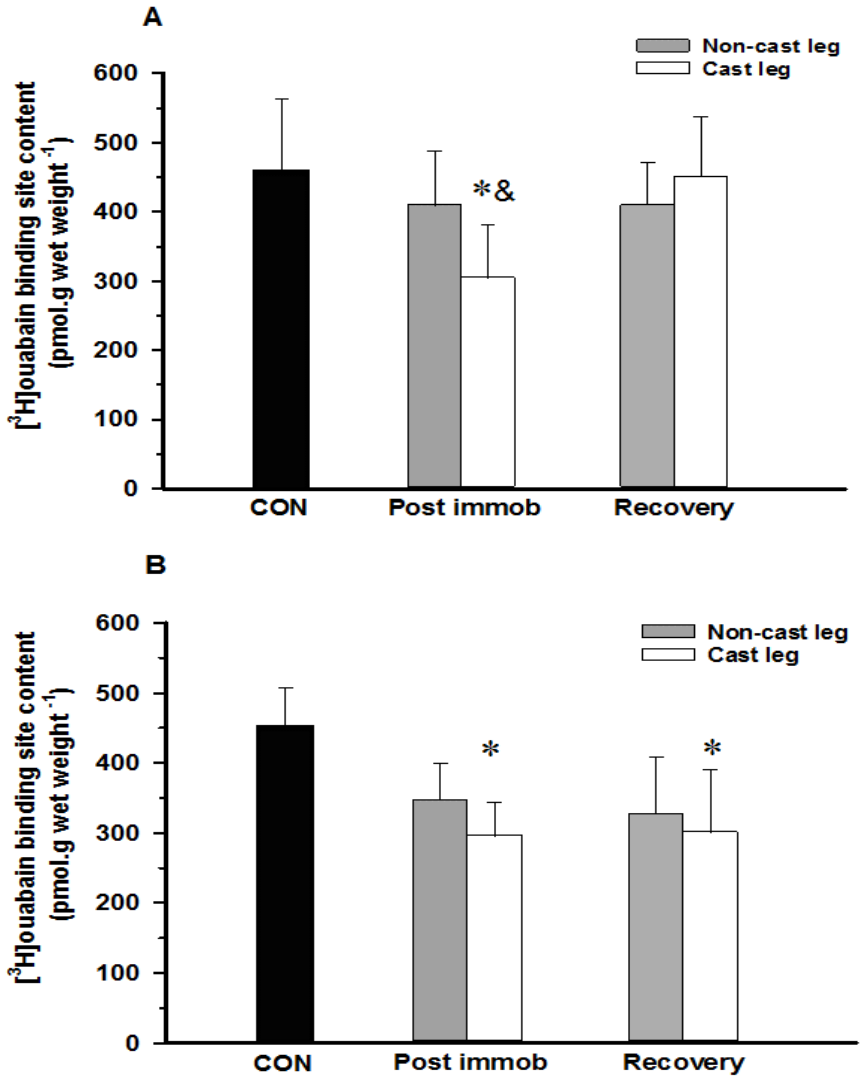

Figure 3.3. Effects of 10 d hindlimb immobilisation and castration in rats on soleus muscle on [^{3}H]ouabain binding site content from (A) sham and (B) castration groups.

CON, non-immobilisation control group; Post immob, 0 d following immobilisation; Recovery, 14 d of recovery after immobilisation. Values are mean ± SD, n = 7 - 8 per group. *cast leg less than control group, $p < 0.001$. & cast leg less than non-cast leg, $p < 0.05$.

3.3.2 NKA isoform abundances

Representative blots for NKA α_1, α_2, α_3, β_1 and β_2 isoforms are shown in Fig 3.4. The NKA β_3 isoform could not be detected, despite attempts at several total protein amounts and using two different antibodies.

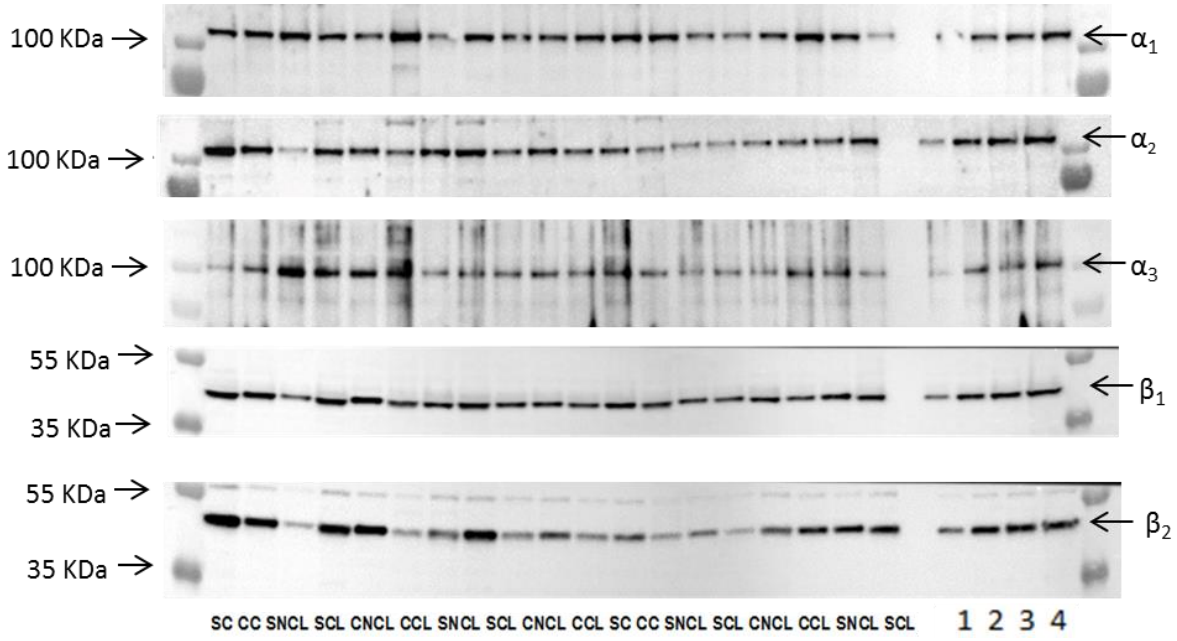

Figure 3.4. Representative immunoblots of NKA α_1, α_2, α_3, β_1 and β_2 isoforms in homogenates of rat soleus muscle.

Values at left indicate molecular weight of bands. Protein bands from left to right are (SC) sham non-immobilised control group, (CC) castration non-immobilised control group, (SNCL) sham non-cast leg, (SCL) sham cast leg, (CNCL) castration non-cast leg, (CCL) castration cast leg. 1, 2, 3 and 4 are calibration curve loaded with 2.5.- 12.5µg whole-muscle crude homogenate. The homogenate was prepared from an equal amount from each sample.

3.3.2.1 NKA α_1 isoform abundance

3.3.2.1.1 Comparison of non-immobilised controls in castration vs sham groups

To determine the effects of testosterone suppression on soleus NKA α_1 isoform abundances independent of hindlimb immobilisation, the non-immobilised control in the castration was compared to non-immobilised control in the sham group. The NKA α_1 isoform abundance did not differ between castration non-immobilised and sham non-immobilised control groups (1.00 ± 0.15 vs 1.10 ± 0.13 a.u., p = 0.228; d = 0.050).

3.3.2.1.2 Within-sham group comparisons

There were no significant differences between groups within-sham comparisons for α_1 (p = 0.876). Post-immobilisation, the NKA α_1 isoform abundance in the cast leg did not differ from either the non-cast leg (p = 0.487; d = 0.313), or non-immobilised control group (p = 0.889; d = 0.712, Fig 3.5A). At 14 d recovery, there were no differences in the NKA α_1 isoform abundance in the cast leg compared to either the non-cast leg (p = 0.569, d = 0.282) or non-immobilised control group (p = 0.895; d = 0.155, Fig. 3.5A).

3.3.2.1.3 Within-castration comparisons

There were no significant differences between groups within-castration comparisons α_1 for (p = 0.754). Post immobilisation, the NKA α_1 isoform abundance in the cast leg did not differ from the non-cast leg (p = 0.442; d = -0.297) or non-immobilised control (p = 0.898; d = 0.05, Fig. 3.5B). At 14 d recovery, the NKA α_1 isoform abundance in the cast leg was not different from the non-cast leg (p = 0.343), and non-immobilised control group (p = 0.411, Fig. 3.5B).

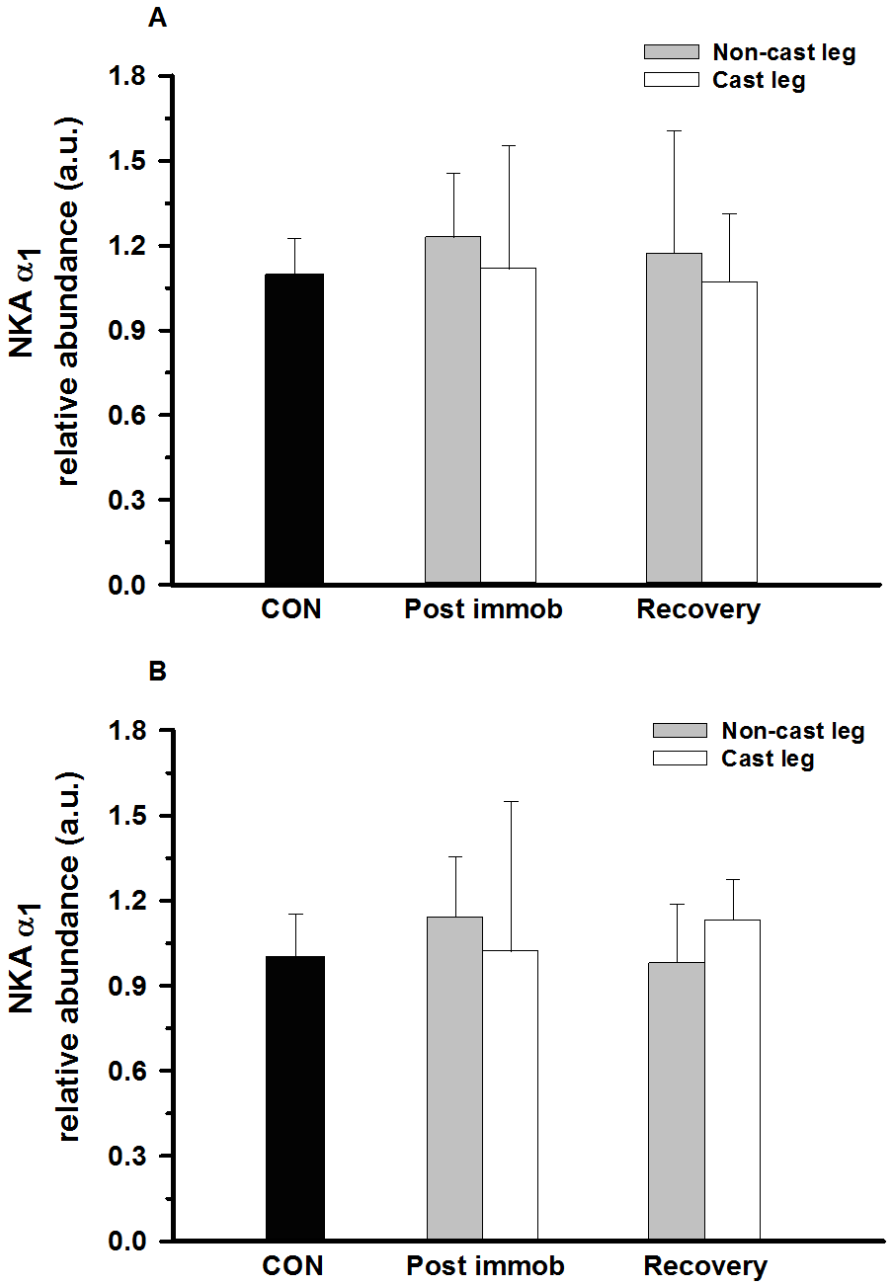

Figure 3.5. Effects of 10 d hindlimb immobilisation and castration in rats on soleus muscle NKA α_1 isoform protein abundance from (A) sham group, (B) castration group.

CON, non-immobilisation control group; Post immob, 0 d following immobilisation; Recovery, 14 d of recovery after immobilisation. Values are mean ± SD in arbitrary units (a.u.), n = 7 - 8 per group.

3.3.2.2 NKA α_2 isoform abundance

3.3.2.2.1 Comparison of non-immobilised controls in the castration vs sham group

To determine the effects of testosterone suppression on soleus NKA α_2 isoform abundances independent of hindlimb immobilisation, the non-immobilised control in the castration was compared to non-immobilised control in the sham group. There was no significant difference in the NKA α_2 isoform abundance between castration non-immobilised and sham non-immobilised control groups (1.37 ± 0.70 vs 1.51 ± 0.66 a.u., $p = 0.695$; $d = -0.193$).

3.3.2.2.2 Within-sham group comparisons

There were no significant differences between groups within-sham group comparisons for α_2 ($p = 0.835$) with considerable variability noted (large SD). Post-immobilisation, the NKA α_2 isoform abundance in the cast leg did not differ from either the non-cast leg ($p = 0.539$; $d = 0.259$) or non-immobilised control group ($p = 0.357$; $d = 0.449$, Fig. 3.6A). At 14 d recovery, there were no differences in the NKA α_2 isoform abundance in the cast leg compared to either the non-cast leg ($p = 0.524$) or non-immobilised control group ($p = 0.339$; $d = 0.60$, Fig. 3.6A).

3.3.2.2.3 Within-castration comparisons

There were significant differences between groups within-castration comparisons for α_2 ($p = 0.010$). Post-immobilisation, the NKA α_2 isoform abundance in the cast leg was ~60% lower than in both the non-cast leg ($p = 0.004$; $d = 1.38$) and non-immobilised control group ($p = 0.004$; $d = 1.37$, Fig. 3.6B). At 14 d recovery, the NKA α_2 isoform abundance remained depressed in the cast leg being ~42% lower than the non-immobilised control group ($p = 0.039$; $d = 0.980$), but was not different from the non-cast leg ($p = 0.812$, $d = 0.340$, Fig 3.6B).

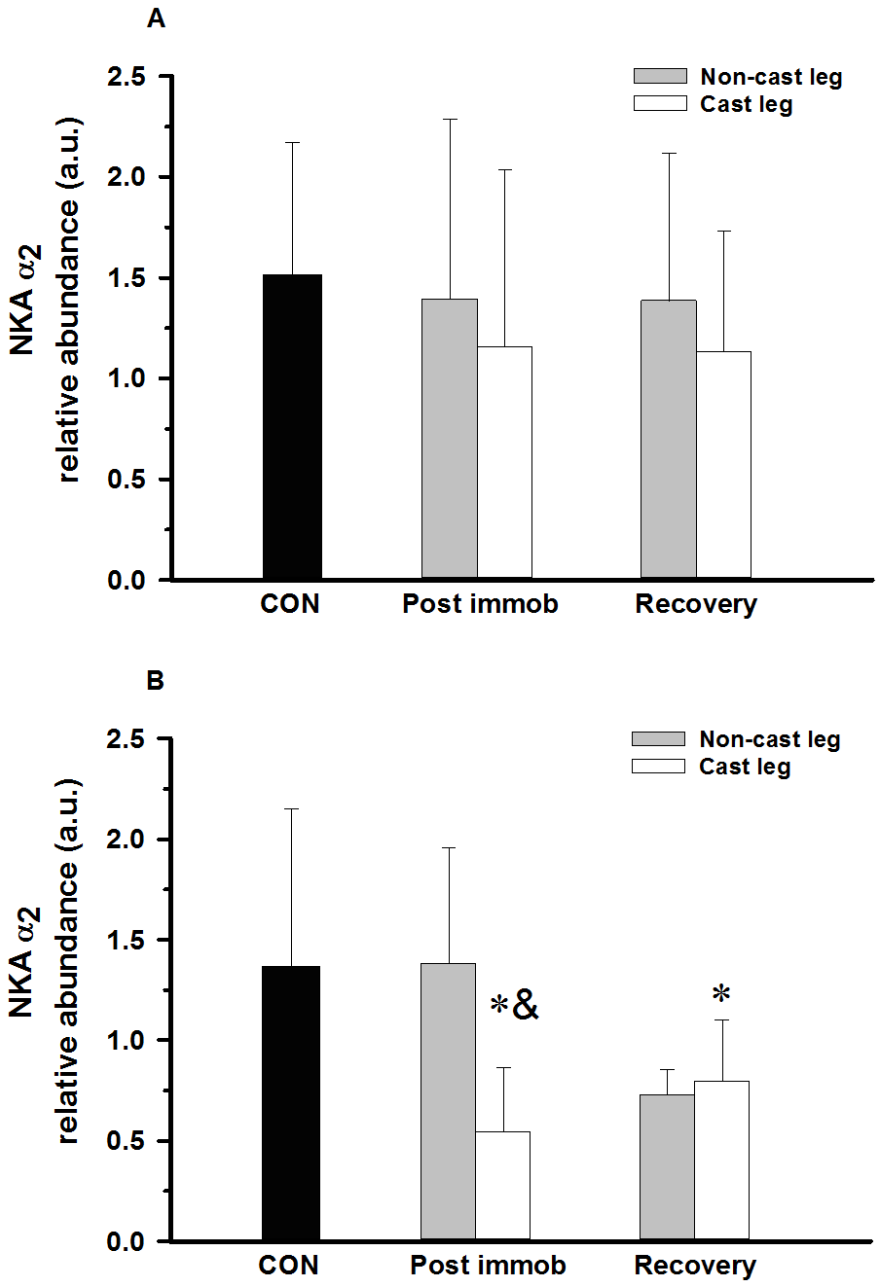

Figure 3.6. Effects of 10 d hindlimb immobilisation and castration in rats on soleus muscle NKA α_2 isoform protein abundance from (A) sham group, (B) castration group.

CON, non-immobilisation control group; Post immob, 0 d following immobilisation; Recovery, 14 d of recovery after immobilisation. Values are mean ± SD in arbitrary units (a.u.), n = 7 - 8 per group. *cast leg less than control group $p < 0.001$, & cast leg less than non-cast leg, $p < 0.05$.

3.3.2.3 NKA α_3 isoform abundance

3.3.2.3.1 Comparison of non-immobilised controls in castration vs non-immobilised sham groups

To determine the effects of testosterone suppression reduction on soleus NKA α_3 isoform abundances independent of hindlimb immobilisation, the non-immobilised control in the castration group was compared to non-immobilised control in the sham group. The NKA α_3 isoform abundance was not different between castration non-immobilised and sham non-immobilised control groups (0.98 ± 0.60 vs 1.15 ± 0.46 a.u., p = 0.255; d = -0.374).

3.3.2.3.2 Within-sham group comparisons

There were no significant differences between groups within sham group comparisons for α_3 (p = 0.990). Post-immobilisation, the NKA α_3 isoform abundance in the cast leg did not differ to either the non-cast leg (p = 0.856; d = 0.51) or non-immobilised control group (p = 0.683; d = 0.160, Fig 3.7A). At 14 d recovery, there was no different in the NKA α_3 isoform abundance in cast leg compared to either the non-cast leg (p = 0.288, d = 115) or non-immobilised control group (p = 0.865; d = 0.222, Fig 3.7.A).

3.3.2.3.3 Within-castration group comparisons

There were no significant differences between groups within castration group comparisons for α_3 (p = 0.641). Post-immobilisation, the NKA α_3 isoform abundance in the cast leg did not differ to either the non-cast leg (p = 0.610, d = 298) or non-immobilised control group (p = 0.491, d = 0.323, Fig. 3.7B). At 14 d recovery, there was no difference in the NKA α_3 isoform abundance in the cast leg compared to either the non-cast leg (p = 0.923, d = 0.075) or non-immobilised control group (p = 0.217, d = 0.580, Fig. 3.7B).

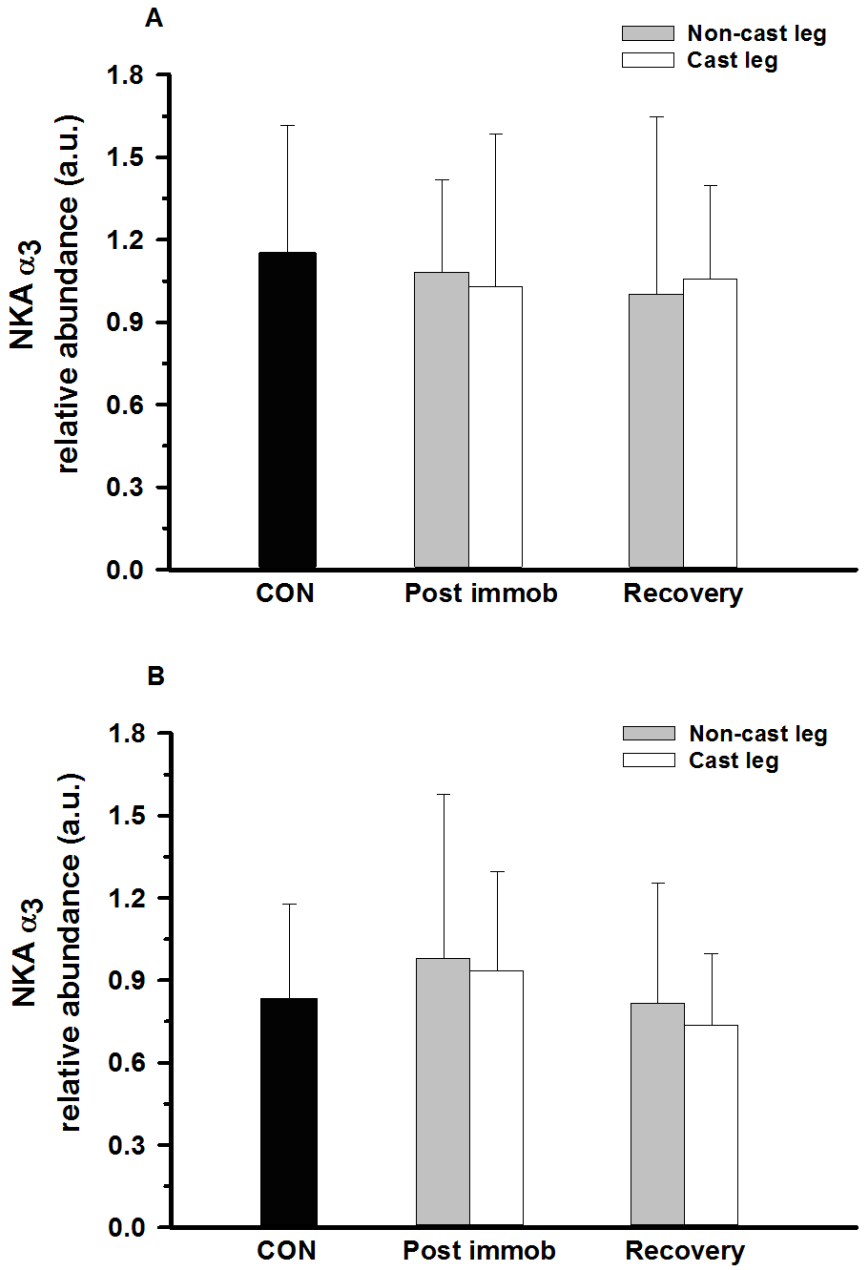

Figure 3.7. Effects of 10 d hindlimb immobilisation and castration in rats on soleus muscle NKA α_3 isoform protein abundances from (A) sham group, (B) castration group.

CON, non-immobilisation control group; Post immob, 0 d following immobilisation; Recovery, 14 d of recovery after immobilisation. Values are mean ± SD in arbitrary units (a.u.), n = 7 - 8 per group.

3.3.2.4 NKA β_1 isoform abundance

3.3.2.4.1 Comparison of non-immobilised controls in castration vs sham groups

To determine the effects of testosterone suppression on soleus NKA β_1 isoform abundances independent of hindlimb immobilisation, the non-immobilised control in the castration was compared to non-immobilised control in the sham group.

The NKA β_1 isoform abundance was not different between castration non-immobilised and sham non-immobilised control groups (0.76 ± 0.43 vs 0.79 ± 0.37 a.u., p = 0.859; d = 0.075).

3.3.2.4.2 Within-sham group comparisons

There were no significant differences between groups within-sham group comparisons for β_1 (p =0.661). Post immobilisation, there was no difference in the NKA β_1 isoform abundance in the cast leg compared to either the non-cast leg (p = 0.920; d = 0.064) or non-immobilised control group (p = 0.858; d = 0.079, Fig. 3.8A). At 14 d recovery, the NKA β_1 isoform abundance in the cast leg did not significantly differ compared to either the non-cast leg (p = 0.141; d = 0.80) or non-immobilised control group (p = 0.628, d = 0.199, Fig. 3.8A).

3.3.2.4.3 Within-castration group comparisons

There were significant differences between groups within-castration group comparisons for β_1 (p = 0.042). Post-immobilisation, the NKA β_1 isoform abundance in the cast leg was 26 % lower than in the non-cast leg (p = 0.018, d = 1.09), but did not differ from the non-immobilised control group (p = 0.321, d = 0.43, Fig. 3.8B). At 14 d recovery, there was no significant differences in the NKA β_1 isoform abundance in cast leg compared to either the non-cast leg (p = 0.427, d = 0.65) or non-immobilised control group (p = 0.251, d = 0.51, Fig. 3.8B).

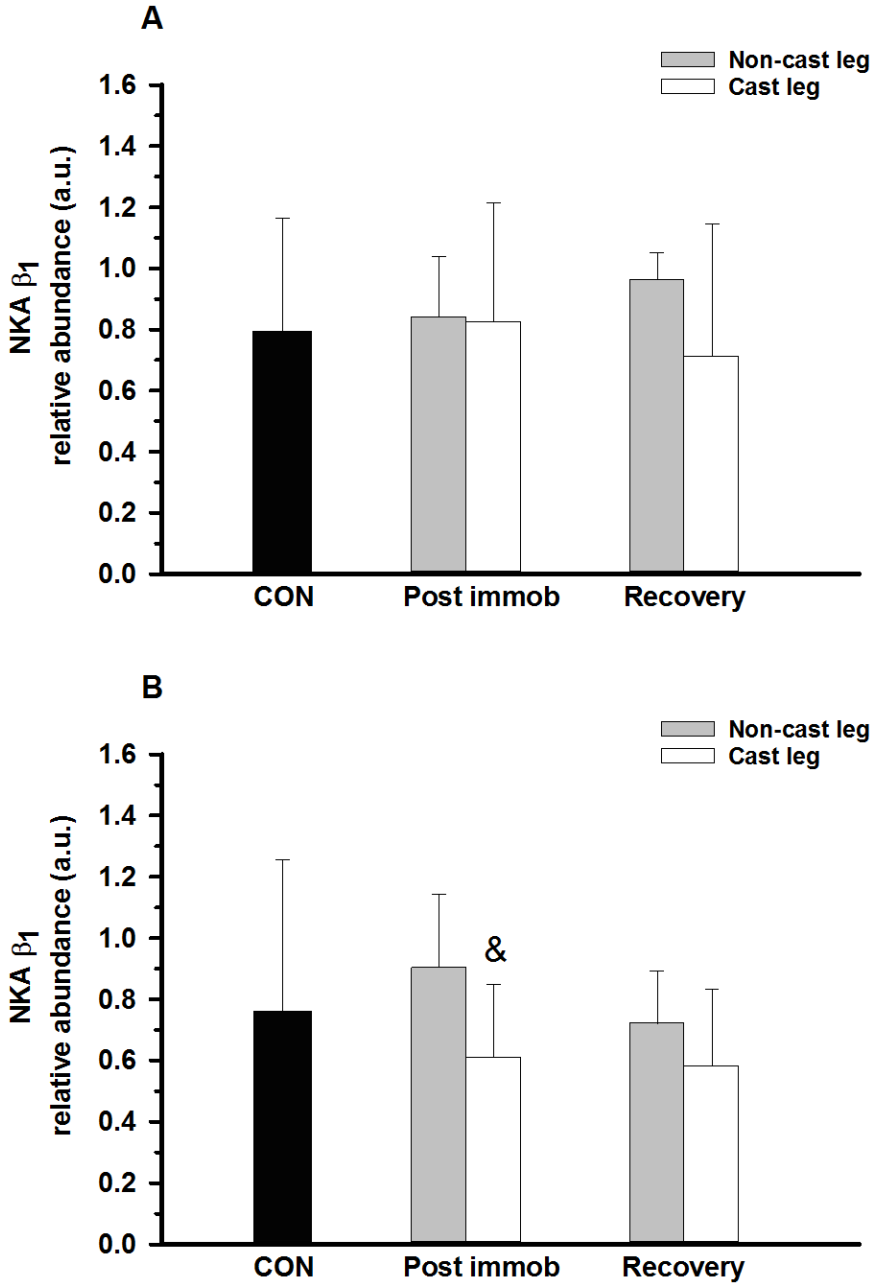

Figure 3.8. Effects of 10 hindlimb immobilisation and castration in rats on soleus muscle NKA β_1 isoform protein abundance from (A) sham group, (B) castration group.

CON, non-immobilisation control group; Post immob, 0 d following immobilisation; Recovery, 14 d of recovery after immobilisation. Values are mean ± SD in arbitrary units (a.u.), n = 7 - 8 per group. & cast leg less than non-cast leg, $p < 0.05$.

3.3.2.5 NKA β_2 isoform abundance

3.3.2.5.1 non-immobilised controls in castration vs sham groups

To determine the effects of testosterone suppression on soleus NKA β_2 isoform abundances independent of hindlimb immobilisation, the non-immobilised control in the castration group was compared to non-immobilised control in the sham group. There was no significant difference in the NKA β_2 isoform abundance between castration non-immobilised and sham non-immobilised controls group (0.90 ± 0.46 vs 0.95 ± 0.55 a.u., $p = 0.358$; $d = -0.098$).

3.3.2.5.2 Within-sham group comparisons

There were no significant differences between groups within-sham group comparisons for β_2 ($p = 0.656$). Post-immobilisation, the NKA β_2 isoform abundance did not differ in the cast leg compared to the non-cast leg ($p = 0.852$, $d = 0.096$) or non-immobilised control group ($p = 0.349$, $d = 0.56$, Fig. 3.9A). At 14 d recovery, there was no difference in the NKA β_2 isoform abundance in cast leg compared to either the non-cast leg ($p = 0.756$; $d = 0.157$) or non-immobilised control group ($p = 0.726$; $d = 0.027$, Fig. 3.9A).

3.3.2.5.3 Within-castration group comparisons

There were significant differences between groups within-castration group comparisons for β_2 ($p = 0.036$). Post-immobilisation, the NKA β_2 isoform abundance was 71% lower than the non-cast leg ($p = 0.004$, $d = 1.60$) and 65% lower than the non-immobilised control group ($p = 0.012$; $d = 0.76$, Fig.3.9B) At 14 d recovery, the NKA β_2 isoform abundance in the cast leg was not different from the non-cast leg ($p = 0.674$; $d = 0.293$) or non-immobilised control group ($p = 0.267$; $d = 0.35$, Fig.3.9B).

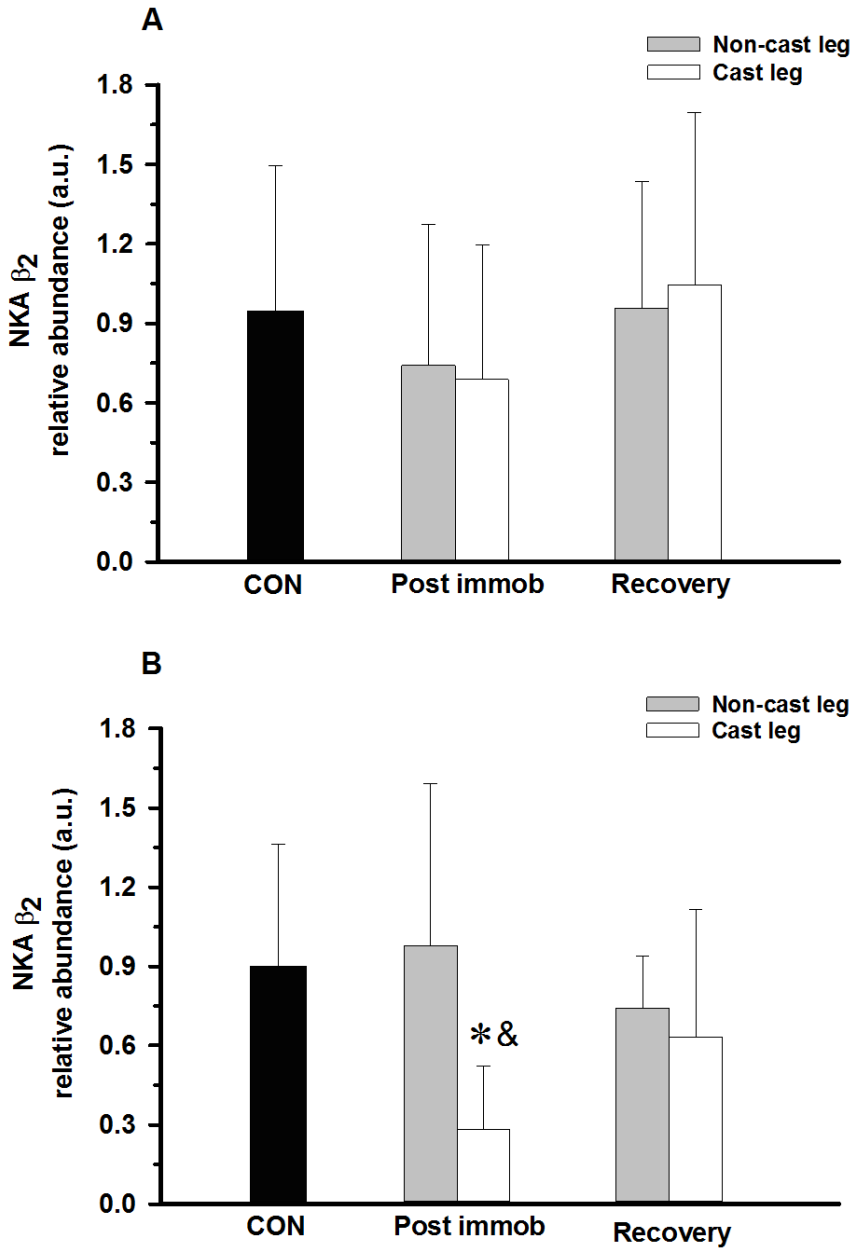

Figure 3.9. Effects of 10 d hindlimb immobilisation and castration in rats on soleus muscle NKA β2 isoform protein abundances from (A) sham group, (B) from castration group.

CON, non-immobilisation control group; Post immob, 0 d following immobilisation; Recovery, 14 d of recovery after immobilisation.Values are mean ± SD in arbitrary units (a.u.), n = 7 - 8 per group. *cast leg less than non-immoblisation control group, $p < 0.05$, & cast leg less than non-cast leg, $p < 0.05$.

3.4 Discussion

This study investigated the effects of hindlimb immobilisation and testosterone suppression via castration on rat soleus muscle [^{3}H]ouabain binding site content and NKA isoform protein abundances. The muscle [^{3}H]ouabain binding site content and NKA isoform abundances were unaffected by castration alone, indicating that 7 d of testosterone suppression did not induce NKA downregulation in rat soleus muscle. When assessed in the sham group, a reduction was found with immobilisation in the [^{3}H]ouabain binding site content, but surprisingly, there were no changes in any of the α_1 - α_3 and β_1 - β_2 isoforms. However, effects were found when the castration was combined with hindlimb immobilisation. Following 10 days of hindlimb immobilisation, the [^{3}H]ouabain binding site content decreased in both the sham and castration groups. However, after 14 days of recovery the [^{3}H]ouabain binding site content had returned to control levels in the sham, but remained depressed in the castration group. This suggests that reduced testosterone may impair recovery of NKA post-immobilisation. The NKA α_2, β_1 and β_2 isoform abundances were not reduced in the sham group, but were reduced following the hindlimb immobilisation in the castration group, but with here that no impairment in recovery at 14 d in the castration group.

3.4.1 Effects of hindlimb immobilisation and testosterone reduction on [^{3}H]ouabain binding site content

Hindlimb immobilisation for 10 d reduced the [^{3}H]ouabain binding site content by 34% in rat soleus muscle. This is consistent with previously reported reductions of the [^{3}H]ouabain binding site content by 20% - 30% in rat soleus muscle (Kjeldsen et al. 1986; Zemkova et al. 1990), 25% in guinea pig gastrocnemius muscles (Leivseth et al. 1992) and 39% in vastus lateralis muscle in sheep (Jebens et al. 1995). This finding is also in line with previous studies in humans undergoing greatly reduced muscle activity, showing

a ~ 23 - 34% decrease in the [^{3}H]ouabain binding site content, in patients with inactive muscle shoulder impingement syndrome (Leivseth and Reikerås 1994), complete spinal cord injury (Ditor et al. 2004) and knee-injury (Perry et al. 2015). Hence, the expected downregulation of NKA as measured by [^{3}H]ouabain binding site content after immobilisation was found, Thus disuse in animal models and in patients undergoing injury greatly restricting mobility all reduce muscle NKA, an effect that may have immediate adverse effects on muscle function (Clausen 2008, 2013b).

Furthermore the [^{3}H]ouabain binding site content had returned to baseline at 14 days after post-immobilisation. In previous studies the [^{3}H]ouabain binding site content had returned to baseline levels after 7 days of recovery following one week of hindlimb immobilisation (Zemkova et al. 1990), and after 4 - 5 weeks of recovery following four weeks of hindlimb immobilisation (Leivseth et al. 1992). Thus the expected recovery following immobilisation was also observed and had occurred by 14 d.

An important initial finding was that castration per se did not reduce the [^{3}H]ouabain binding site content in rat soleus muscle. The most important and novel finding is that testosterone suppression via castration reduced the [^{3}H]ouabain binding site content by 34% with immobilisation and prevented its recovery after hindlimb immobilisation. In the sham group, the [^{3}H]ouabain binding site content decreased similarly by ~34% in the cast leg, but had returned to control levels after 14 days of recovery. In contrast, it remained significantly depressed in the castration group by 34% after 14 days of recovery when compared to the control group. This decline in NKA may be functionally important as it may potentially impair the regulation of Na^+ efflux and K^+ influx and membrane potential with muscle contractions, and potentially contribute to reduced muscle function via inexcitability and fatigue (Clausen 2003a). The downregulation of the [^{3}H]ouabain

binding site content in this study was associated with a ~30% reduction in both muscle mass and force compared with non-immobilised control animals ($p<0.001$, $p<0.05$) respectively, and a ~50% ($p<0.001$) reduction compared with the non-cast leg (Lin et al. 2016), Appendix 6 Muscle mass and force were not reported after 14 days of recovery.

3.4.2 Effects of hindlimb immobilisation and of testosterone reduction on NKA isoform abundances

The NKA α_2 isoform did not significantly change in the sham group following hindlimb immobilisation, despite the substantial reduction of [^{3}H]ouabain binding in the same group, however, a moderate effect size was found after immobilisation even after 14 days of recovery. This lack of decrease in NKA α_2 was surprising, since the α_2 isoform has a strong affinity with ouabain binding in rat muscle. The lack of significant change in the α_2 isoform may be due to the semi-quantitative and variable nature of western blot analysis, making it difficult to detect the changes. This finding appears to contrast with previous studies where short-term hindlimb suspension in the rat for 6 hours and for 1 - 3 days, decreased the electrogenic activity of the NKA α, which is defined as measuring the ouabain sensitive change in resting membrane potential, which is generated by electrogenic NKA transport and is a sensitive, real-time assay of NKA activity in intact skeletal muscle cells (Kravtsova et al. 2015; Kravtsova et al. 2016). However, this was a much short time course as well as distinctly different measures. In inactive human muscle after injury, the α_2 isoform abundance was 63% lower in the knee-injured leg than the non-injured leg, which was associated with ~20% lower muscle NKA content (Perry et al. 2015). The α_2 isoform abundance was unchanged with unilateral lower limb suspension (ULLS), which was consistent with unchanged muscle NKA content (Perry et al. 2016). However, their results were based on a similarly small sample size (six

subjects), which may have contributed to the inability to detect a significant change in muscle NKA content and isoforms. One additional interpretation is that simply being able to move the muscles/hindlimb may have been sufficient activity to prevent the anticipated decline in the NKA content with ULLS, as shown inpartial spinal cord injury patients (Boon et al. 2012).

In contrast with the lack of change in sham group, this study demonstrated a downregulation of the NKA α_2 isoform in the castration group following hindlimb immobilisation and this remained significantly depressed after 14 days of recovery. This coincided with reductions in the [^{3}H]ouabain binding site content. This 60% decrease in NKA α_2 isoform protein abundance was however, double that of the [^{3}H]ouabain binding site content decline (32%). The disparity between the [^{3}H]ouabain binding site content and α_2 isoform findings may reflect the methodological differences used for each measurement. Western blot analysis is based on a standardised total protein concentration from homogenate muscle, whilst the [^{3}H]ouabain binding technique is based on intact portions of muscle tissue. The [^{3}H]ouabain binding site content also reflects binding of functional pump (i.e. $\alpha\beta$ complexes) whereas the western blot measures all isoforms present in the tissue. The α_2 is the most abundant of the α subunits in rat soleus muscles, comprising ~ 80 - 85% of α subunits (He et al. 2001; Hansen and Clausen 1988). Furthermore, the α_2 isoform plays a major role in Na^+ and K^+ transport in working muscle (DiFranco et al. 2015). The partial global knockout of α_2 caused impaired contractile force in mouse EDL muscle (Lingrel et al. 2003), whilst muscle specific α_2 knockout reduced muscle strength, increased muscle fatigability and reduced exercise performance in mice (Radzyukevich et al. 2013). Hence this reduction in the α_2 isoform with immobilisation and castration may contribute to impaired muscle excitability and contractility.

A clear finding from this study is that the NKA α_1 isoform was not affected by hindlimb immobilisation or testosterone suppression via castration. This is the first time this has been shown for the more extended immobilisation periods over which [^{3}H]ouabain binding site content has typically been measured. This is an important finding given that in rat soleus muscle, the α_1 isoform represents 15 - 25% of the NKA α isoforms (Hansen 2001). Thus observed reductions in the [^{3}H]ouabain binding site content with immobilisation were not underestimating overall decreases due to missing additional reductions in other isoforms, given α_1 resistance to ouabain binding. The unchanged α_1 isoform protein abundance with immobilisation is also consistent with unchanged α_1 isoform electrogenic activity after short-term hindlimb immobilisation for 6 hours and 1-3 days in rat soleus muscle (Kravtsova et al. 2015; Kravtsova et al. 2016).

This is the first study showing that the NKA α_3 isoform abundance is also not affected by hindlimb immobilisation or testosterone reduction. It is believed that the NKA α_3 isoform protein abundance is very low in rat skeletal muscle (Clausen 2003a). In human vastus lateralis muscle, the NKA α_3 isoform abundance in muscle homogenates did not change after either chronic knee-injury or with unilateral lower limb suspension (Perry et al. 2015; Perry et al. 2016). However, in type I single muscle fibres the NKA α_3 isoform abundance decreased after 23 d of unilateral lower limb suspension (Perry et al. 2016). In skeletal muscle, the functions and abundance of the NKA α_3 remain unknown, but the absence of change in α_3 isoform indicates these are unlikely to play major role adverse effects of immobilisation or castration.

This study also demonstrated NKA β_1 and β_2 isoform downregulation with immobilisation in the castration, but not in the sham group. There were large variations in the measurements of β_1 and β_2 isoforms, possibly reflecting the small sample size and

low statistical power. In the cast leg, the β_1 isoform decreased in the castration group by 26% compared to non-cast leg, whereas the β_2 isoform was 71% and 65% lower than in the non-cast leg and control group, respectively. This suggests a reduction in both $\alpha_2\beta_1$ and $\alpha_2\beta_2$ complexes account for the reduction in [^{3}H]ouabain binding site content with immobilisation. In the rat the β_1 isoform is the most abundant in muscles rich in slow-twich fibres such as in soleus muscle, whilst the β_2 is more abundant in muscles rich in fast twitch fibres such as in EDL muscle (Hundal et al. 1994; Thompson and McDonough 1996; Fowles et al. 2004; Zhang et al. 2006). Despite the β_1 and β_2 isoforms differing between muscle fibre types in rat muscle, the function of each β isoform in skeletal muscle NKA regulation remains poorly understood. The β isoform is essential for regulating NKA activity, and transporting and stabilising the movement of the α isoform from the endoplasmic (sarcoplasmic) reticulum to the plasma membrane (Lavoie et al 1997; Scheiner-Bobis 2002; Geering 1990; Mcdonough et al. 1990; Chow and Forte 1995). Therefore, downregulation in NKA β_1 and β_2 isoform abundances with immobilisation and castration are likely to important, and may reflect reduced skeletal muscle NKA activity thereby potentially impacting adversely on muscle fatigue (Lavoie et al. 1997).

Given the soleus muscle comprises ~ 78% of type I fibres (Dimov[1] and Dimov 2007), it is not clear whether the findings noted in this study were specific to type I fibres and/or also occurred in to type IIa fibres; hence, further investigation on possible different effects on muscle fibres types is required.

The mechanisms which may cause a decrease [^{3}H]ouabain binding site content in skeletal muscle are not understood. During electrical stimulation, the NKA activation in rat soleus muscle was associated with an increase in intracellular [Na^+] (Everts and Clausen 1992; Nielsen and Clausen 1997). Thus it is likely that the downregulation of NKA with

immobilisation may be linked with less increases in the intracellular [Na^+] as result of reduced muscle contractions. The possible mechanism of effects of testosterone reduction on NKA are also likely via disturbances in muscle ion regulation, as testosterone is a regulator of intracellular Na^+. Castration induced a decrease in the Na^+-dependent transport in rat ventral prostate cells which was increased after injection with testosterone (1.mg) for 24 h (Lao et al. 1993). Testosterone treatment (10^{-7} M) for 20 min elevated the K^+ -dependent dephosphorylation in ventral prostate in castrated rats (Farnsworth 1970). Incubation of human radial artery in 100 μM testosterone also increased K^+ efflux through K_{ATP} channels, resulting in vasorelaxation (Seyrek et al. 2007). Here no effect of testosterone was observed on [^{3}H]ouabain binding site content, or any α or β isoforms in the non-immobilisation control muscle. A likely mechanism is via α_2 isoform specific relationships. Given the large size of α_2 pool in rat soleus muscle, about ~ 80-85% of the NKA α isoforms (He et al. 2001; Hansen and Clausen 1988). Thus any decrease in α_2 isoform may cause downregulation in the overall NKA. The electrogenic activity of the NKA α_2 reduced with brief immobilisation in rat soleus muscle (Kravtsova et al. 2015; Kravtsova et al. 2016), which decreased membrane depolarisation (Krivoi et al. 2008). The electrogenic contribution of α_2 isoform was 57% of the total NKA and decreased to 16% after 3 d of suspension in rat soleus muscle (Krivoi et al. 2008). The reduced muscle [^{3}H]ouabain binding site content and α_2 isoform abundances might differ to any depression in NKA activity, but this cannot be confirmed since the activity of NKA was not measured in this study due to the limitations of current methods for measurement of NKA activity (section 2.2.5). The mechanism underlying reduced α_2 abundance thus remains unclear.

Another possible mechanism for decreased [^{3}H]ouabain binding site content is via impairment or lesser activity of biochemical signalling pathways via the non-ion transducing role of NKA. This may occur as result of direct-protein interactions between NKA and its neighbouring proteins, which triggers a signalling cascade culminating in decreased NKA gene transcription (Aperia et al. 2016; Xie and Askari 2002; Xie and Cai 2003). However, since this has so far been shown to be specific to the α_1 isoform, this therefore seems unlikely since no changes were observed with immobilisation and castration in α_1 isoform abundance. However, this requires further specific research.

3.4.3 Conclusions

The [^{3}H]ouabain binding site content in rat soleus muscle was reduced with immobilisation in both the sham and castration groups, and returned to control levels after 14 days in the sham group, but remained depressed in the castration group. Hence testosterone suppression via castration did not modify the reduction in the [^{3}H]ouabain binding site content with immobilisation, but prevented its subsequent recovery when already low. This was likely due to changes in the α_2 isoform abundance, which was reduced post-immobilisation and castration and also failed to recover after 14 days. The skeletal muscle NKA α_2, β_1 and β_2 isoform abundances were not reduced with immobilisation only (sham), but were decreased with immobilisation in the castration group. Thus it is likely that reduction in both $\alpha_2\beta_1$ and $\alpha_2\beta_2$ complexes occurred after immobilisation and testosterone suppression. Neither hindlimb immobilisation nor testosterone suppression affected the skeletal muscle NKA α_1 and α_3 isoform abundances. These results might be important in understanding recovery after immobilisation, especially in men likely to have lowered testosterone levels compared to healthy individuals such as in prostate cancer with androgen deprivation therapy, type 2 diabetes

and the elderly. These findings open a new promising area of research into the effects of testosterone on skeletal muscle NKA, requiring further study in humans.

Chapter 4: Effects of sprint training on skeletal muscle NKA content and isoform abundance in humans.

4.1 Introduction

During high intensity muscle contractile activity such as in sprinting, the capacity to minimise disturbances in $[Na^+]$ and $[K^+]$ gradients across the muscle cell membranes may play an important role in delaying muscle fatigue (Clausen 2013a; McKenna et al. 2008; Sejersted and Sjøgaard 2000). The NKA is the key protein that regulates cellular Na^+ and K^+ exchange in skeletal muscle, and thus is essential for maintaining muscle function during intense contractions. The NKA comprises a catalytic α and a regulatory β subunit, which in skeletal muscle are expressed as α_1, α_2 and α_3, and as β_1, β_2 and β_3 isoforms (Juel et al. 2013; Clausen 2013b; Murphy et al. 2004).

Repeated sprint exercise is a commonly used training paradigm for many team sport athletes (Spencer et al. 2005; Bishop et al. 2011), and has been found to contribute to a wide range of metabolic and morphological adaptations in human skeletal muscle (Ross and Leveritt 2001; Harmer et al. 2006). Sprint training in recreationally active individuals comprising repeated 30 s maximal work-bouts increased skeletal muscle NKA content by 8 - 16%, improved plasma K^+ regulation and enhanced sprint exercise performance (McKenna et al. 1993; Harmer et al. 2006). However, the effects of sprint training utilising sprint bouts of 30 s or less on the individual NKA isoforms in skeletal muscle are not well understood. Sprint training comprising repeated 30 s bouts at 130%VO_{2max} over eight weeks in untrained healthy subjects led to an increase in the NKA α_2 isoform by 68% and in β_1 (percentage not reported), but did not increase the α_1 isoform (Mohr et al. 2007). This suggested an important functional role of NKA α_2 upregulation in muscle ion homeostasis during intense intermittent exercise (Mohr et al. 2007). In contrast, sprint

training comprising repeated 30 s running bouts at 90-95% max running speed over three to four weeks in endurance trained runners, increased the NKA α_1 isoform by 29%, but with no changes in α_2 and β_1 isoforms (Iaia et al. 2008). Thus, it cannot be confirmed that adaptations in the NKA α_2 are vital during intense intermittent exercise training. Whilst two earlier studies demonstrated an increased muscle NKA content with sprint training (McKenna et al. 1993; Harmer et al. 2006), neither determined which α isoforms were in fact increased. Of the two subsequent studies that did investigate NKA isoforms, one showed increased α_2 and β_1, with α_1 unchanged (Mohr et al. 2007), and the other an increased α_1 with α_2 and β_1 isoforms unchanged (Iaia et al. 2008). Importantly neither measured the NKA content. Thus, it is not clear whether the induced increase in NKA content is due to which α NKA isoforms and whether this is accompanied by an increase in β_1 isoforms. Furthermore, the effects of 30 s repeated sprint training on skeletal muscle NKA α_3, β_2 and β_3 isoform abundances are not known. Hence this study investigated the effects of sprint training combining for the first time each of skeletal muscle NKA content with α and β isoform abundances.

The aim of this study was to investigate the adaptions of skeletal muscle NKA content and α_1 - α_3, β_1 - β_3 isoform abundances, and to determine which isoforms were upregulated to account for the anticipated upregulation of skeletal muscle NKA content, following 7 weeks of repeated 30 s sprint exercise training, in healthy young adults. It was hypothesised that an increased skeletal muscle NKA content after sprint training would be associated with upregulation of each of the skeletal muscle NKA α_1, α_2 and β_1 isoform abundances.

4.2 Methods

4.2.1 Participants and overview

Fifteen healthy, active, but not well trained young adults (11 males, 4 females) gave written informed consent prior to participating in this study (age 24.6 ± 5.3 yr, height 1.83 ± 0.7 cm, body mass 80.19 ± 15.13 kg, mean±SD). Participants were randomly allocated into either a sprint training (n=8, 2 females and 6 males, ST) or a control (n=7, 2 females and 5 males, CON) group. G*Power version 3.1.7 software was used to calculate the required sample size. Calculation of the sample size was based on findings from previous study and expected muscle NKA content difference, of 300 pmol/g ww for control, assuming a 16% increased content in sprint training (McKenna et al.) and with a SD within each group of 30 pmol/g ww, yields the following power analysis. Thus with n=15 in each group a=0.05, the expected power was 95 %.

Each participant underwent a vastus lateralis muscle biopsy at rest, prior to and following either a 7 week ST intervention or CON period, with a post-training biopsy taken 72 h after the final training session. Participants in CON were asked to continue their regular daily activities for this period. The study was approved by the Victoria University Human Research Ethics Committee.

4.2.2 Sprint Training Program

The sprint training program was based on an earlier described program (McKenna et al. 1993). The training protocol comprised three training sessions per week for 7 weeks and was performed on a Velotron cycle ergometer (Dynafit Pro – RacerMate Cycle, Seattle, Washington), with 2 - 3 days recovery between training sessions. A standardised 10 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 comprised repeated 30 s maximal cycling efforts, interspersed with 4 min of recovery (rest or light cycling at 30 W) between each sprint bout during weeks 1 - 4; the recovery time was then reduced by 30 s each successive week until week 7. Progressive overload was implemented into the training program by increasing the number of bouts from four during week 1, to six during week 2, and to eight bouts during week 3, and finally to ten bouts during weeks 4 - 7. The training was conducted in a laboratory under supervision. The training was supervised by Trever Farr and was part of a longer study with measurement of arterial and venous blood sampling and [K^+] measures (Farr et al unpublished data)

4.2.3 Muscle samples.

Each participant underwent a vastus lateralis muscle biopsy at rest, prior to and after 72 h after the final training session or CON period. After injection of local anaesthetic into the subcutaneous skin and fascia (1% Xylocaine, Astra Zeneca), an incision was placed in skin overlying the vastus lateralis muscle. A muscle sample (~100 - 120mg) was extracted from the mid-thigh using a Bergström biopsy needle with suction. Muscle was rapidly frozen in liquid nitrogen and stored at -80 °C for later analyses of NKA content and isoform protein abundances.

4.2.4 [^{3}H]-ouabain binding site content

Analysis of the skeletal muscle [^{3}H]-ouabain binding site content to determine muscle NKA content, was performed as described in Chapter 3, except for the final correction factor which was adjusted for human muscle using 1.13, as previously described (Petersen et al., 2005).

4.2.5 Western blotting

The NKA α_1 - α_3 and β_1 - β_3 isoform protein abundances were measured using the western blotting technique, as described in Chapter 3. Antibodies used were as described except for NKA β_1 isoform where the Na^+,K^+-ATPase β_1 (monoclonal, Thermo Scientific # MA3-930) antibody was used.

4.2.6 Statistics

All data were analysed using a one-tailed paired t-test (pre vs post) since increases were hypothesised with ST and the ST and CON groups were analysed separately. Data was assessed for normality using the Shapiro-Wilk test. A log transformation was used to ensure a normal distribution of data before subsequent analysis. Statistical analyses were conducted using SPSS version 24. Effect size was calculated using Cohen's *d*, where <0.2, 0.2 - 0.5 and 0.5 - 0.8 and >0.8 are considered trivial, small, moderate and large, respectively (Cohen 1988). Pearson's product-moment correlation coefficient was used to examine correlations between NKA content and NKA α_1 and α_2 isoforms. Data were presented as mean ± standard deviation (SD), and statistical significance was accepted at $P < 0.05$. All figures were created using SigmaPlot 13 (Systat Software, Inc).

4.3 Results

4.3.1 [^{3}H]ouabain binding site content

The muscle [^{3}H]ouabain binding site content tended to be higher (28 %) after ST but this was not significant, although a moderate effect size was found ($p = 0.063$, $d = 0.487$), with no changes in CON ($p = 0.172$, Fig 4.1). However, inspection of individual data revealed that six of the eight participants did respond to training with an increased NKA content. If statistically analysed separately only for these six participants then the NKA content was increased by 46.8 % following ST (314.0 ± 86.3 vs. 461.0 ± 88.5 pmol.g wet weight^{-1}). There were no significant correlations between muscle NKA content and NKA α_1 ($r = 0.46$, $p = 0.457$) or α_2 ($r = 0.021$, $p = 0.480$) isoform abundances.

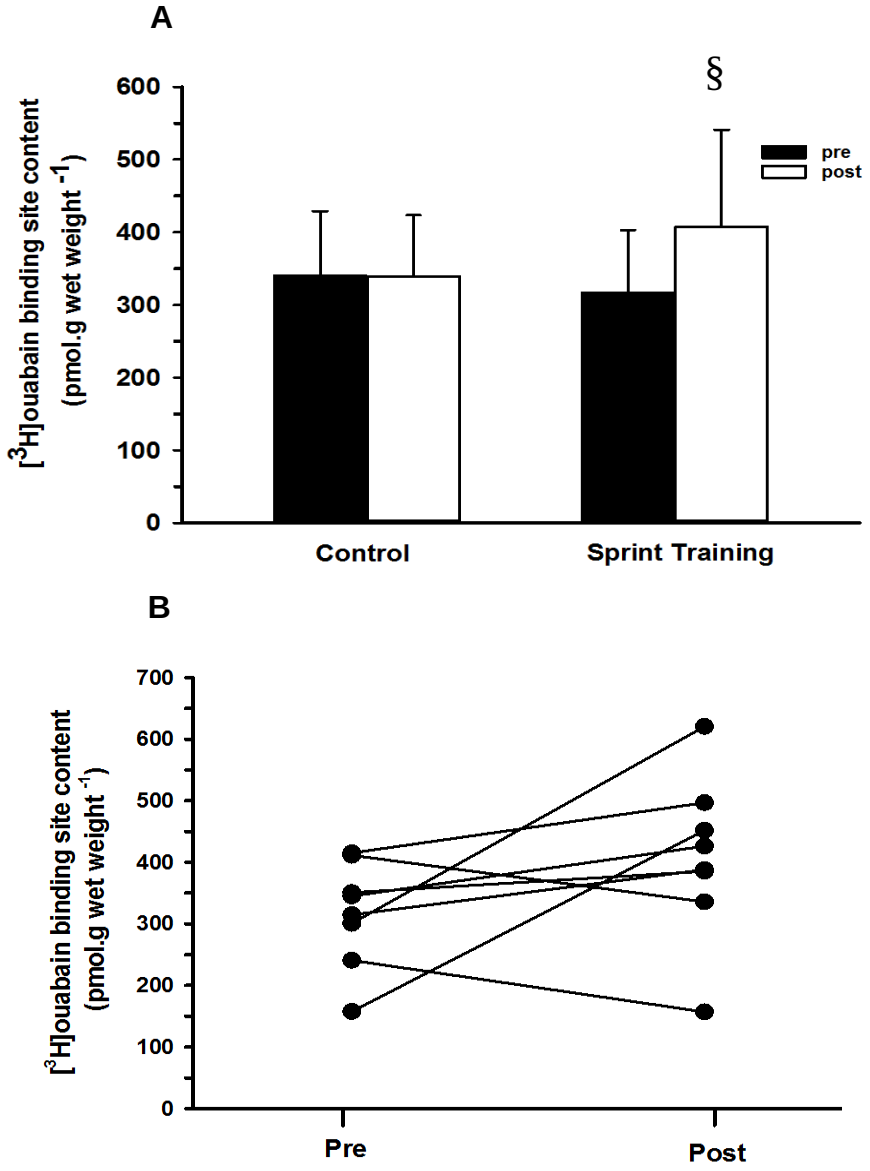

Figure 4.1. Skeletal muscle [^{3}H]ouabain binding site content in healthy untreaind young adults A) before and after 7 wk of sprint training and B) individual responses in the ST training to training.

Data are presented as mean ± SD, n = 8 Sprint training, n = 7 control. § tended to be greater than pre training (p = 0.063)**.**

4.3.2 NKA isoform abundance

Representative blots for NKA α_1, α_2, α_3, β_1 and β_2 are shown in Fig 4.2. Neither the NKA α_3 or β_3 isoforms could be detected in these individuals, despite attempts at several total protein amounts and using two different antibodies.

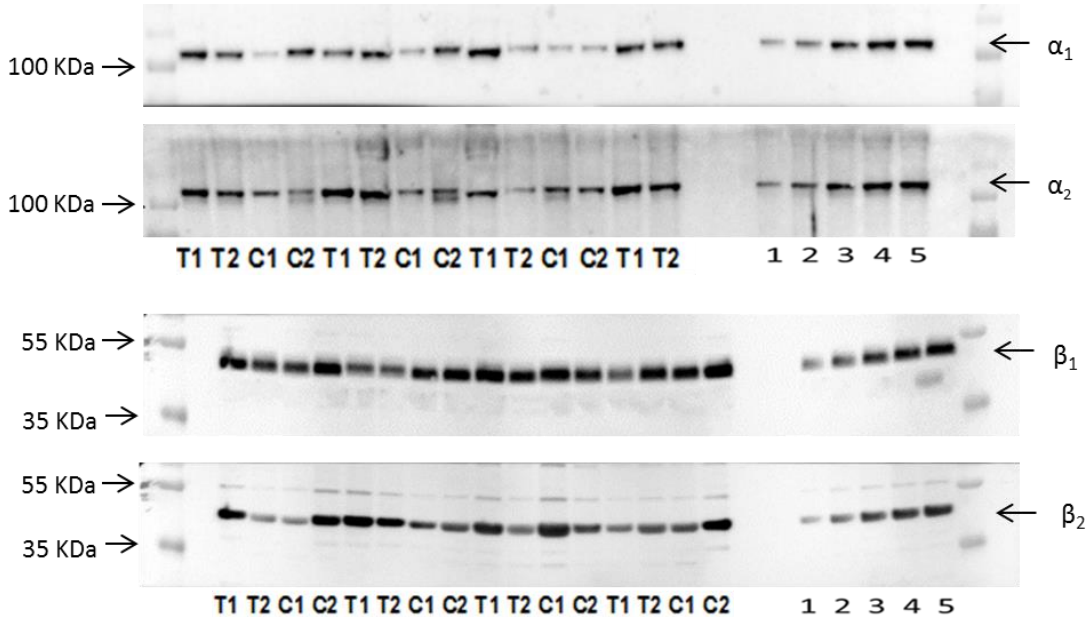

Figure 4.2. Representative immunoblots of NKA α_1, α_2, β_1 and β_2 isoforms in homogenates of human vastus lateralis muscle.

Values at left indicate molecular weight of bands. Protein bands from left to right are (T1) pre-training, (T2) post-training (C1) pre-control, (C2) post-control. 1, 2, 3 and 4 are calibration curve loaded with 2.5 - 12.5µg whole-muscle crude homogenate. The homogenate was prepared from an equal amount from each sample.

4.3.2.1 Muscle NKA α isoform abundances

No significant differences were found after ST in NKA α_1 ($p = 0.268$, $d = 0.34$), or α_2 ($p = 0.341$, $d = 0.21$) isoform protein abundances, with no differences also detected in CON ($p = 0.462$, $d = 0.045$ and $p = 0.112$, $d = 0499$, respectively, Fig 4.3). Due to the small differences in control group α_2 isoform between post and pre (0.23 a.u), the delta changes were calculated. There were no significant differences found in dalta α_2 isoform between control group and training group (-0.32 ± 0.45 vs 0.86 ± 0.33, $p = 0.336$).

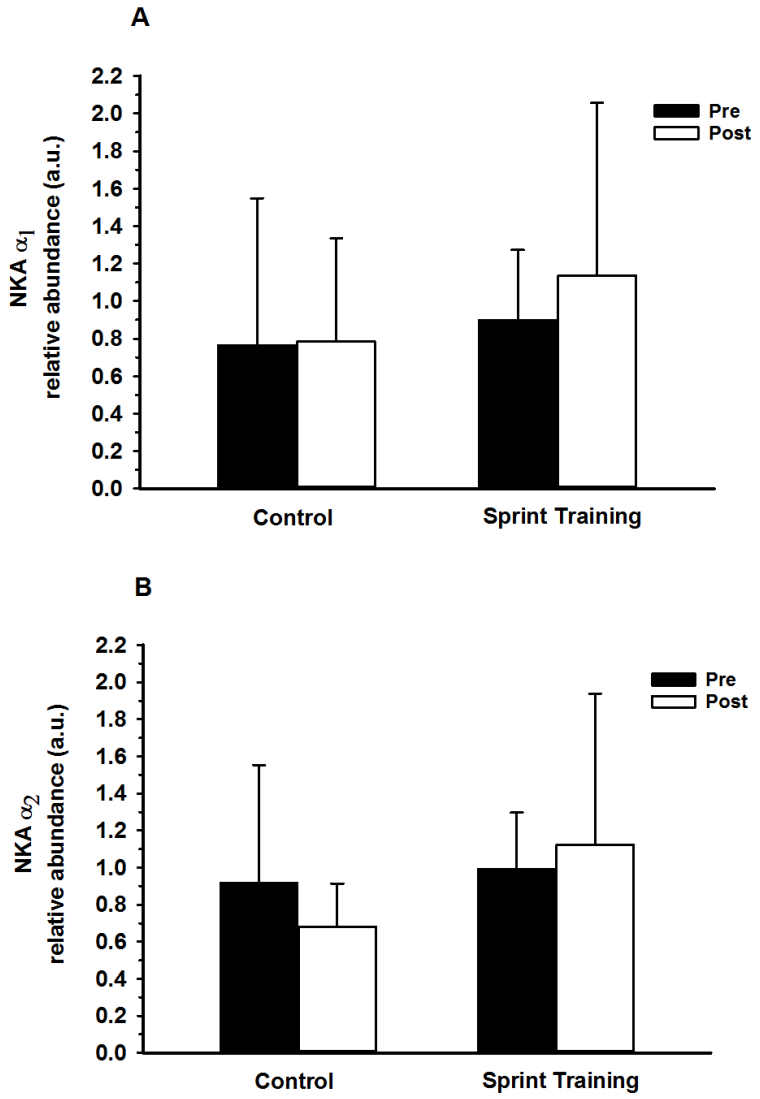

Figure 4.3. Skeletal muscle NKA isoform protein relative abundance for (A) α_1 and (B) α_2 before and after 7 wk of sprint training in healthy, untrained young adults.
Data are presented as mean ± SD in arbitrary units (a.u.), n = 8 sprint training, n = 7 control.

4.3.2.2 Muscle NKA β isoform abundances

There were no significant differences found after ST in NKA β_1 ($p = 0.375$, $d = 0.17$) or β_2 ($p = 0.424$, isoform protein abundances, with no differences also in CON ($p = 0.228$, $d = 0.34$ and $p = 0.135$, $d = 0.281$, respectively, (Fig 4.4). Due to the small differences in control group β_1 and β_2 isoforms between post and pre (0.11 a.u and 0.16 a.u, respectively), the delta changes was calculated. There were no significant differences found for delta β_1 and delta β_2 between control group and training group (-0.10 ± 0.36 vs 0.08 ± 0.59, $p = 0.481$, and (-0.16 ± 0.35 vs -0.05 ± 0.76, $p = 0.742$, respectively).

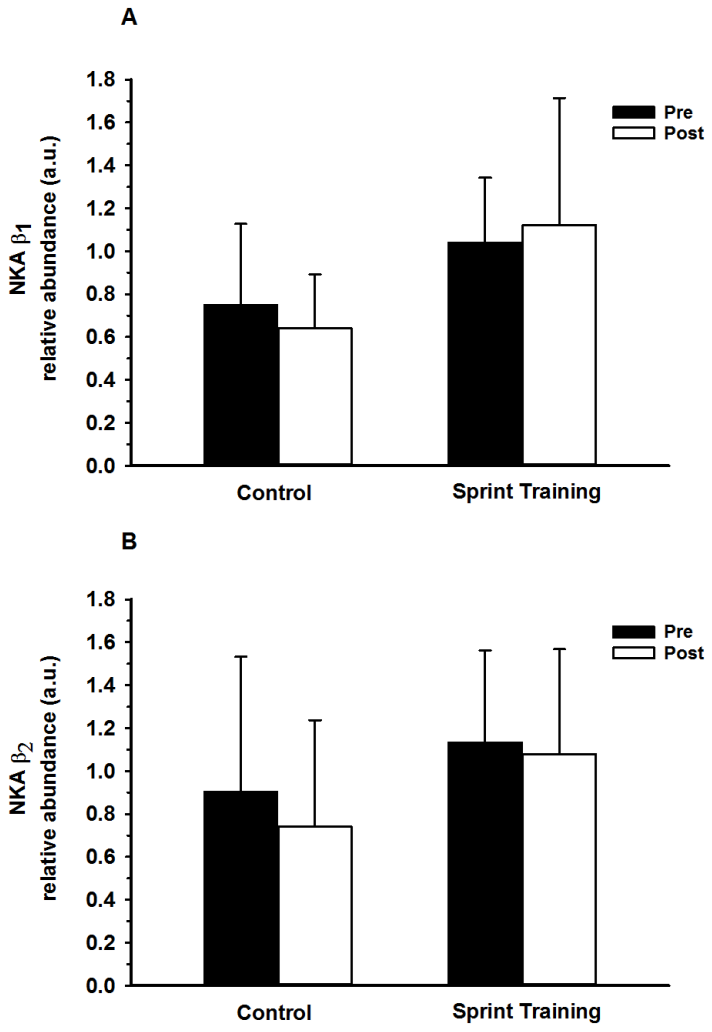

Figure 4.4. Skeletal muscle NKA isoform protein relative abundance for (A) β_1 and (B) β_2 before and after 7 wk of sprint training in healthy, untrained, young adults

Data are presented as mean ± SD in arbitrary units (a.u.), n = 8 sprint training, n = 7 control

4.4 Discussion

This study investigated the effects of sprint training comprising repeated maximal 30s bouts on skeletal muscle NKA content and NKA isoform abundances in healthy human adults. Contrary to expectations, sprint training did not increase any of the muscle NKA content or NKA α_1, α_2, β_1 or β_2 isoform protein abundances, although a tendency to increase NKA content was observed ($p<0.07$) reflecting higher values post-training in 6 of the 8 participants. The non-significant change in skeletal muscle NKA content was however consistent with also no changes detected in α_1 and α_2 NKA isoform abundances and no significant correlations were found between these variables.

4.4.1 The effects of sprint training on skeletal muscle NKA content

That muscle NKA content did not significantly change after seven weeks of sprint exercise training was surprising, given increased muscle NKA content findings from two previous utilising an identical training protocol and similar number of participants (McKenna et al. 1993; Harmer et al. 2006). This is even more surprising given that an increase in NKA content is a common finding after many different types of exercise training (Table 2.1), even after only six days of submaximal cycling training (Green et al. 2004 Green et al, 1993). It is unlikely that the lack of effect was due to the ST training having an insufficient total load to stimulate increases in muscle NKA content, since the same training protocol previously clearly increased muscle NKA content (McKenna et al. 1993; Harmer et al. 2006). The lack of response to ST is unlikely to be attributed to the total amount of activity before training. The participants were untrained but recreationally active and had no difference in NKA content from control; also the VO_{2peak} of participants in this study (3.40 ± 0.75 $L.min^{-1}$) was also consistent with a lower physical activity status. However, the lack of significant upregulation in NKA

content was consistent with no increase found in the 30 s sprint peak power (pre-train 959 ± 306 vs post-train 984 ± 331) or mean power (pre-train 370 ± 167 vs post-train 375 ± 126) outputs after ST in these participants (Farr et al. unpublished data). This suggests that a major reason underlying lack of significant adaptation in muscle NKA in this study was that participants failed to improve with ST. The reasons for this are not clear. It is possible for these participants that an even greater training stimulus may have been required for these individuals

4.4.2 The effects of sprint training on skeletal muscle NKA isoform abundances

Since in this study 30 s sprint training did not affect any of the NKA isoform abundances, it is not possible to resolve the discrepancy between previous studies as to which α isoforms were increased. Thus the unchanged α_1 isoform abundance after sprint training is consistent with one previous study (Mohr et al. 2007), but contrasts another study showing an increased NKA α_1 isoform by ~29% after sprint training (Iaia et al. 2008). The α_1 NKA isoform is believed to play a housekeeping role in skeletal muscle, with high affinity for both Na^+ and K^+ ions (Clausen 2003a). The lack of change in the NKA α_2 isoform after sprint training is consistent with previous findings in one (Iaia et al. 2008), but contrasts another (Mohr et al. 2007) after a similar style of 30s sprint training. This study also found no effect of 30 s sprint training on NKA β_1 isoform abundances. The lack of change in the NKA β_1 isoform after sprint training is consistent with previous findings in one study (Iaia et al. 2008), but contrasts another after a similar style of 30s sprint training (Mohr et al. 2007). This is the first study to examine the effect of 30 s sprint training on the NKA β_2 isoform abundance, finding no adaptability of β_2. This finding is similar to previous studies reporting unchanged NKA β_2 isoform abundance after high intensity cycling training exercise in already well trained cyclists (Aughey et

al 2007) and after endurance cycling training in healthy untrained adults (Benziane et al 2011). For reasons that remain unclear the NKA α_3 and β_3 isoforms were attempted at several total protein concentrations using two different antibodies, but could not be detected. Similar difficulties in detection were previously reported in our laboratory for the β_3 isoform (Perry et al 2015) (Atanasovska unpublished data). Due to similar ouabain affinity, an increased [^{3}H]ouabain site content with training could reflect increased α_1, α_2, and/or α_3 isoforms separately, or combined.

The lack of significance in muscle NKA content and isoforms after training may be due to a type II error, since the result was based on a small number of participants (n = 8), with low statistical power of (0.29), (0.25), (0.25) (0.28) (0.26) for NKA content and α_1, α_2, β_1 and β_2 isoforms, respectively. There was a tendency to higher NKA content after training, with a moderate effect size ($p = 0.063$, $d = 0.487$). Of the eight participants in this study, six showed an increase in muscle NKA content. The small number of participants were also associated with a high variability in [^{3}H]ouabain binding site content and NKA isoform western blot results. Thus analysing results from an increased number of participants would most likely have been beneficial.

In this study the NKA isoform abundances were measured using whole muscle homogenates. However, repeated sprint training comprising "all-out" maximal effort with shorter duration bouts (6s) induced fibres type-specific upregulation of the NKA β_1 isoform abundance in type IIa fibres, but did not influence other isoform abundances (Wyckelsma et al. 2015). Thus the results from this study can therefore not necessarily be generalised to muscle fibre types. Hence measuring the NKA isoform abundances in single fibres is needed to clarify the effects of repeated 30 s bouts of sprint training on NKA isoform abundances.

Finally it is possible that repeated 30 s bouts of sprint training are more effective when combined with other types of training to increase total load, in order to stimulate increases in NKA isoform abundances. Combining 30 s sprint runs with small-sided soccer drills in elite soccer players increased muscle NKA α_2 isoform abundance by 15% (Thomassen et al. 2010). Combining 30s running training with aerobic high and moderate intensity training in trained endurance runners increased muscle NKA α_2 by 68% and β_1 isoform abundances (Bangsbo et al. 2009). Combining 30s running training with strength and aerobic training in trained endurance runners elevated muscle NKA β_1 isoform abundance by 27% (Vorup et al. 2016). Further research is required to determine whether this is the case.

4.4.3 Conclusions

Sprint exercise training with repeated 30 s bouts tended to increase skeletal muscle NKA content. The overall lack of significant change was consistent with no significant differences found in the key NKA α isoform abundances, α_1 and α_2. However, when data from the six responders out of eight was analysed, NKA content was significantly increased with training. There were also no significant changes in the NKA β_1 and β_2 isoform abundances following 30 s of sprint exercise training. Further research is required with larger sample size to reduce the impact of measurement variability in NKA content and NKA isoform abundances.

Chapter 5: Effects of resistance training on skeletal muscle NKA content and isoform abundance

5.1 Introduction

The NKA is vital in maintaining steep transmembrane [Na^+] and [K^+] gradients across the cell, and thus in skeletal muscle has an essential role in maintaining excitability and contractions (Clausen 2003a). In skeletal muscle, the NKA comprises three catalytic α isoforms (α_1 - α_3) and three regulatory β isoforms (β_1 - β_3) (Murphy et al. 2004; Wyckelsma et al. 2015). In skeletal muscle, the α_1 is important for basal Na^+/K^+ exchange and affects muscle strength (Radzyukevich et al. 2013; Lingrel et al. 2003), whereas the α_2 has an important role in Na^+ and K^+ transport in working muscle, enhancing exercise performance and resisting fatigue (DiFranco et al. 2015; Radzyukevich et al. 2013). Thus, the regulation of NKA content and isoform abundances is vital for muscle function (Clausen 2010; Sejersted and Sjøgaard 2000).

Previous studies have shown that skeletal muscle NKA content and abundance of some NKA isoforms are upregulated by different types of exercise training, including sprint and endurance training (McKenna et al. 1993; Green et al. 2007; Benziane et al. 2011; Thomassen et al. 2010; Green et al. 2008; Iaia et al. 2008; Mohr et al. 2007; Bangsbo et al. 2009). Resistance training is well known to increase both muscle size and strength, which is a primary goal for athletes involved in many different types of sport (Naclerio et al. 2013; Hoff and Helgerud 2004; Fleck and Kraemer 2014). Interestingly, the effects of resistance training on muscle NKA content and isoform abundances remains unclear. Two studies that have investigated the effects of resistance training on muscle NKA content have showed inconsistent results. Following seven weeks of resistance training in healthy untrained participants, the NKA content was increased by 16% (Green et al.

1999b). However, in well trained athletes; three months of resistance training given once, twice or three times a week each failed to increase the NKA content (Medbø et al. 2001); However, when these groups were combined, the NKA content was increased by 15% (Medbø et al. 2001). One limitation was that this later finding was based on participants who undertook different amounts of training. Only one study has examined the effects of resistance training on skeletal muscle NKA isoforms, finding that 6 weeks of resistance training in one limb in healthy, untrained participants increased each of NKA α_1, α_2 and β_1 isoforms, by 37%, 21% and 33%, respectively, in the trained compared to the untrained leg (Dela et al. 2004). This suggests that increases in NKA content with resistance training are due to increases in both α_1 and α_2 isoforms. However, this not confirmed since no study has combined measures of [^{3}H]ouabain binding site content and NKA isoforms to examine which isoforms are associated with the adaptation of NKA content following resistance training. Also, the possible effects of resistance training on NKA α_3, β_2 and β_3 isoforms remain unclear.

Therefore, this study investigated which NKA isoforms (α_1 - α_3, and β_1 - β_3) were upregulated along with an anticipated increase in skeletal muscle NKA after resistance exercise training in healthy young adults. Based on the previous findings that resistance training increased muscle NKA content (Green et al. 1999b; Medbø et al. 2001) and NKA α_1, α_2, and β_1 isoforms in older individuals (Dela et al. 2004), it was hypothesised that the muscle NKA content and the protein abundance of α_1, α_2, and β_1 isoforms would be increased following resistance exercise training. Since there is little data on adaptation of α_2, β_1 and β_1 isoforms it wasn't possible to form a clear hypothesis for these isoforms.

5.2 Methods

5.2.1 Participants and overview

Twenty-one healthy young adult males that were active but not well trained (n = 16, resistance training and n = 5 control) gave written informed consent prior to participating in this study, which was approved by the Victoria University Human Research Ethics Committee. The physical characteristics of the participants were; age: 22.9 ± 4.6 yr; height: 1.80 ± 0.70 m; body mass: 85.1 ± 17.8 kg (mean±SD). Participants in the training group were assisted with correct techniques and familiarised with the resistance training (RT) program. Each participant underwent a resting vastus lateralis muscle biopsy prior to and following a 7 week RT intervention or control period. Participants in the control group were asked to continue with their regular daily activities for this period.

This study was part of a larger collaborative study which included investigation of the effects of cold water immersion (CWI) recovery after resistance training on exercise performance, expression of genes and proteins involved in the acute and chronic adaptation to exercise (Petersen et al unpublished data). The performance data therefore do not, form part of this study. Half of the participants in the training group underwent CWI during recovery. CWI was initiated within 5 minutes of completing each workout. CWI participants were immersed to their umbilicus for 15 minutes in 10°C water in a recovery pool (iCool Sport, Australia), whilst non-CWI participants sat for 15 minutes in ambient room air (22±0.8°C). Since were no significant differences or any tendencies found between the CWI and non-CWI groups for any of NKA content or NKA α_1, α_2, β_1 and β_2 isoform abundances (statistical power for these power variable were, 0.77, 0.77, 0.81, 0.75 and 0.79, respectively), the results from both groups were pooled into a single RT group.

5.2.3 Resistance Training Program

The RT protocol comprised three training sessions per week for 7 weeks. A standardised 5 min warm up on a cycle ergometer was conducted prior to every training session. Training loads were determined during a familiarisation session performed prior to commencing the training program. Participants performed three sets of 10 - 12 repetitions of each exercise, interspersed with 60 - 90 s of recovery between sets. To induce progressive overload, the training weight was incremented by ~5% when a participant completed 12 repetitions of the three total sets. The training program involved upper and lower body exercises and comprised: back squat, leg raise, leg press, bench press, incline bench press, lat pulldown, lunge, dumbbell shoulder press, dumbbell bicep curl, lying triceps extensions, bent-over row, upright row, barbell biceps curl, dips and sit-ups. Thus 5 out of the 13 exercises were likely to have directly recruited the vastus lateralis muscle. The training was conducted in a laboratory under supervision of Dr Petersen.

5.2.4 Muscle sampling.

Participants in the RT and CON groups underwent a resting vastus lateralis muscle biopsy prior to and after the 7 weeks training period, with the latter biopsy in RT taken 48 - 72 h after the final training session. The muscle biopsy procedure was identical to that described in Chapter 4.

5.2.5 [^{3}H]-ouabain binding site content

Analysis of skeletal muscle NKA content was determined by the [^{3}H]-ouabain binding site content assay, performed as described in Chapter 4.

5.2.6 Western blotting

NKA α_1 - α_3, β_1 - β_3 isoform protein abundances were measured using the western blotting technique as described in Chapter 3.

5.2.7 Statistics

All data were analysed using a one-tailed paired t-test (pre vs post) with RT due to the hypothesised increase in NKA with RT and CON groups analysed individually. Data were assessed for normality using the Shapiro-Wilk test, and all data were normally distributed. Statistical analyses were conducted using SPSS version 24. Effect size was calculated using Cohen's *d*, where <0.2, 0.2 - 0.5 and 0.5 - 0.8 and >0.8 are considered trivial, small, moderate and large, respectively (Cohen 1988). Correlations between NKA content and NKA isoforms α_1 and α_2 were analysed using Pearson's product-moment correlation coefficient. Data are presented as mean ± standard deviation (SD) and statistical significance was set at $P < 0.05$. All figures were created using SigmaPlot 13 (Systat Software, Inc).

5.3 Results

5.3.1 Skeletal muscle NKA content

The muscle NKA content increased by 12% after RT ($p = 0.012$, $d = 0.50$), with no significant changes in CON ($p = 0.271$, $d = 0.157$, Fig 5.1). Increases were seen in 15 of the 16 participants after RT. The NKA content tended to be significantly correlated to α_1 isoform abundance ($r = 0.387$, $p = 0.077$), but not with α_2 isoform abundance ($r = 0.101$, $p = 0.360$).

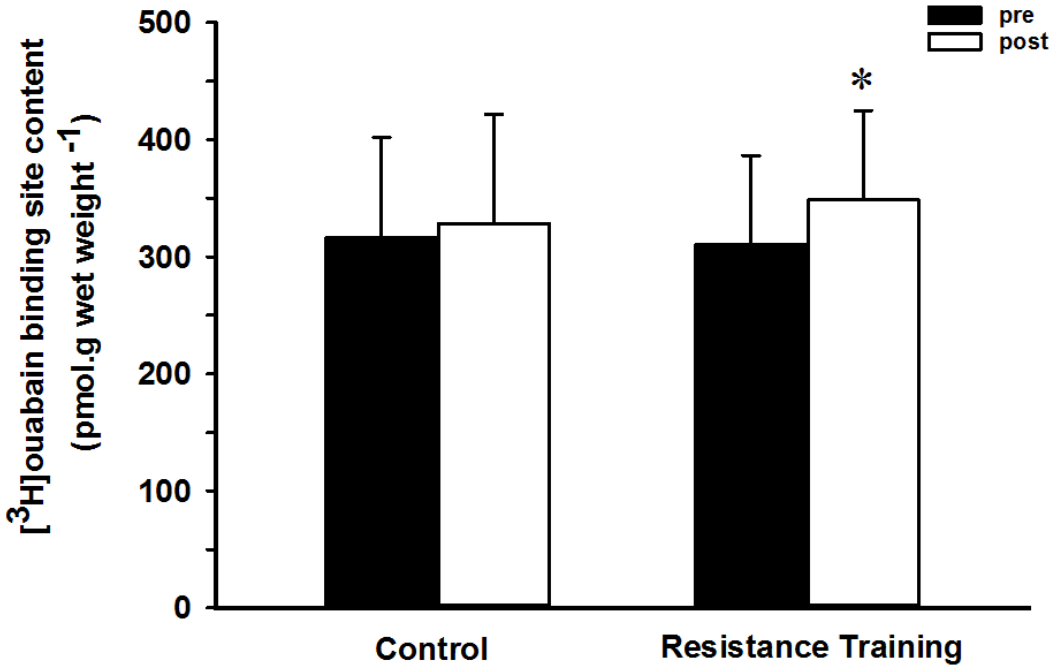

Figure 5.1. Skeletal muscle NKA [^{3}H]ouabain binding site content before and following 7 wk of resistance training in healthy young adults.

Data are presented as mean ± SD. *post-training greater than pre-training, $p < 0.05$, n = 16 resistance training, n = 5 control).

5.3.2 Skeletal Muscle NKA isoform abundances

Representative blots for NKA α_1, α_2, β_1 and β_2 are shown in Fig 5.2. Neither the NKA α_3 or β_3 isoforms could not be detected, despite attempts at several total protein amounts and using two different antibodies.

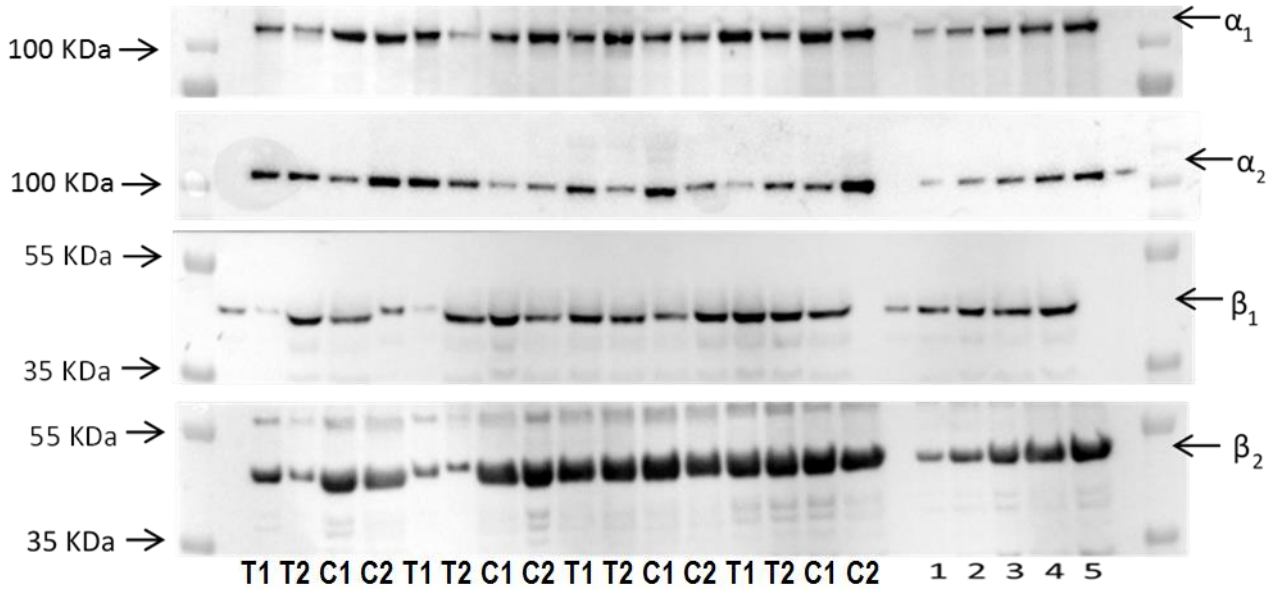

Figure 5.2. Representative immunoblots of NKA α_1, α_2, β_1, and β_2 isoforms in homogenates of the human vastus lateralis muscle.

Values at left indicate molecular weight of bands. Protein bands from left to right are (T1) pre-training, (T2) post-training (C1) pre-control, (C2) post control. 1, 2, 3 and 4 are calibration curve loaded with 2.5 - 12.5µg whole-muscle crude homogenate. The homogenate was prepared from an equal amount from each sample.

5.3.2.1 Skeletal Muscle NKA α isoform abundances

The muscle NKA α_1 isoform increased by 32% ($p = 0.013$, $d = 0.850$), and the α_2 isoform by 10% ($p = 0.001$, $d = 0.362$) following 7 weeks of RT, with no significant differences in CON ($p = 0.174$, $d = 0.48$ and $p = 0.33$, $d = 0.083$, respectively, Fig 5.3). Increases in α_1 isoform were seen in 14 of the 16 participants after RT, whilst all 16 participants showed an increase in α_2 isoform.

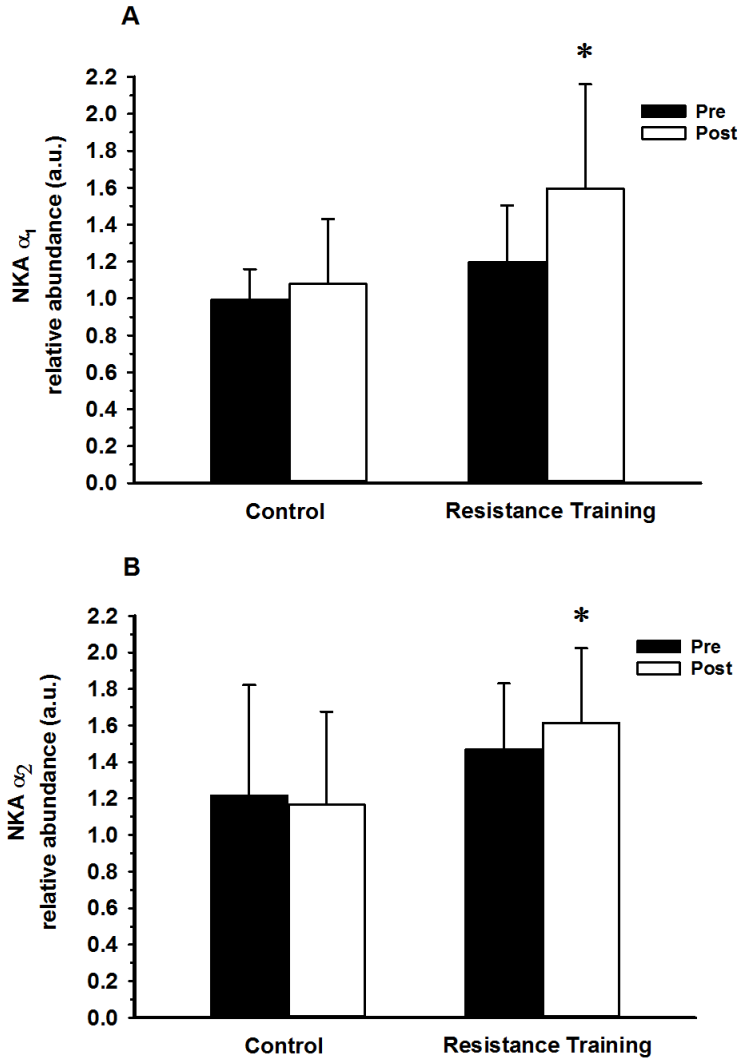

Figure 5.3. Skeletal muscle NKA (A) α_1 and (B) α_2 isoform abundances before and following 7 wk of resistance training in healty young adults.

Data are presented as mean ± SD in arbitrary units (a.u.). *post-training greater than pre-training, $p < 0.05$, n = 16 resistance training, n = 5 control).

5.3.2.2 Skeletal muscle NKA β isoform abundances

The muscle NKA β_1 (p = 0.180, d = -0.252) and β_2 (p = 0.221, d = -0.582) isoforms were not significantly changed following 7 weeks of RT and there were also not significant changes in CON (p = 0.254, d = 0.095 and p = 0.287, d = 0.132, respectively, Fig 5.4).

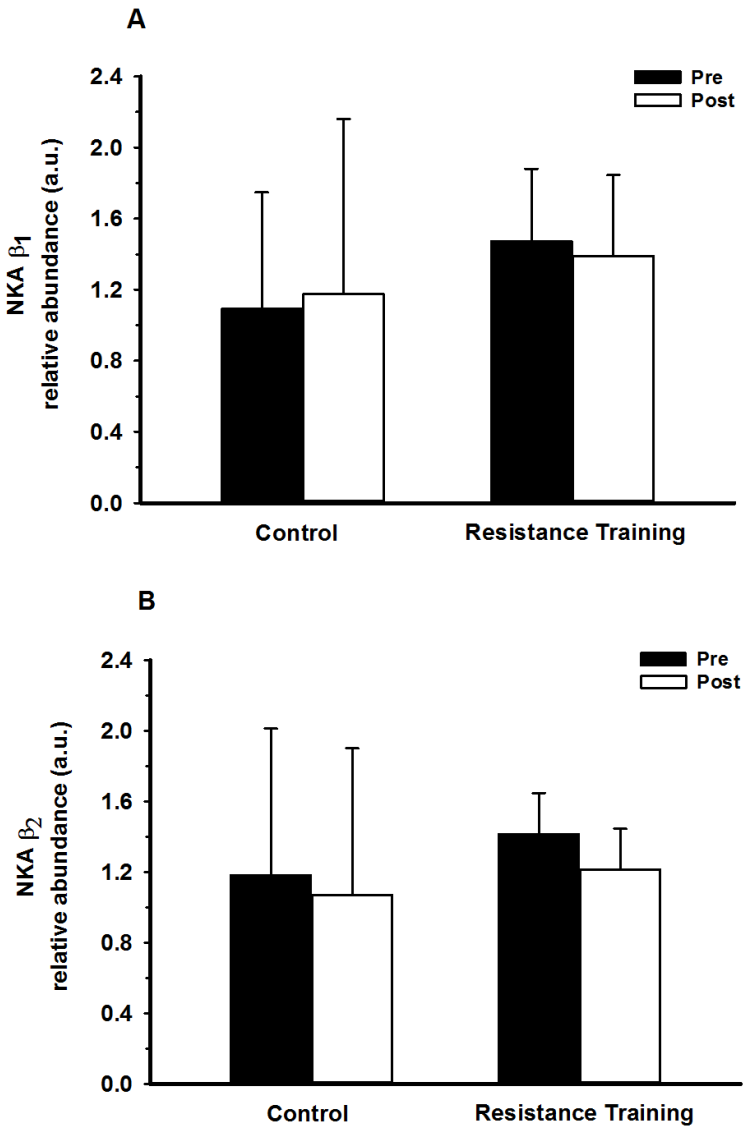

Figure 5.4. Skeletal muscle NKA (A) β_1 and (B) β_2 isoform abundances before and following 7 wk of resistance training in health young adults.

Data are presented as mean ± SD in arbitrary units (a.u.) n = 15 resistance training, n = 5 control.

5.4 Discussion

This study investigated for the first time the effects of resistance training on both NKA content and NKA isoform abundances in skeletal human muscle. Findings indicate that RT induced increases in the muscle NKA content and that this was due to increased abundances of the both of the major NKA α isoforms (α_1 and α_2) and were mostly accounted for by the increased α_2 isoform. Furthermore, RT was not accompanied by any increases in NKA β_1 or β_2 isoform abundances.

5.4.1 The effects of resistance exercise training on skeletal muscle NKA content

The first finding was that 7 weeks of RT increased muscle NKA content by 12%, consistent with the 16% increase reported following 7 weeks of resistance training in healthy untrained subjects (Green et al. 1999) and the 15% increase after 3 months RT in pooled data from a group of highly trained athletes who undertook different amounts of RT (Medbø et al. 2001). Of the 16 participants, 14 showed an increase with RT in muscle NKA content, hence it can be concluded that resistance exercise training clearly results in an upregulation of NKA content. This finding is also in broad agreement with other studies investigating different training models that typically induced increases in NKA content of 8 - 16% following sprint training (McKenna et al. 1993; Harmer et al. 2006) and ~16% following endurance training (Madsen et al. 1994; Evertsen et al. 1997), However, the finding is inconsistent with the lack of increase after sprint training in the previous chapter in this thesis.

Skeletal muscle contractions induce an influx of Na^+ into muscle cells and an efflux of K^+ from the cells, and a high NKA capacity is needed to regulate these ion fluxes to sustain muscle function and excitability (Clausen 2010; Sejersted and Sjøgaard 2000). This capacity in skeletal muscle can be measured by the [^{3}H]ouabain binding site content

(Clausen 2013b). Thus the upregulation of NKA content with training is consistent with a vital requirement to maintain excitability or declines and thus delay or minimise muscle fatigue, and as a consequence, improve exercise performance (McKenna et al. 1993; Harmer et al. 2006). The upregulation of NKA content with RT in this study was associated with improvements in one repetition maximum (1RM) by 10% in bench press and by 39% in the leg press, with the upper and lower body lean mass also increased after training by ~4.6%, Appendix 5 (Petersen et al, unpublished data).

5.4.2 The effects of resistance training on skeletal muscle NKA isoform abundances

The major finding in this study was that RT induced increases of 32% and ~10% in NKA α_1 and α_2 isoform abundances, respectively. Of the sixteen participants, 14 showed an increase with RT in both muscle NKA content and the α_1 isoform, whilst all 16 participants showed an increase also in the α_2 isoform. This is consistent with the only other study to have investigated RT effects on NKA isoforms, which reported an increase in NKA α_1 and α_2 isoform abundances by 37% and 21%, respectively (Dela et al. 2004). Together this data demonstrates for the first time that increases in the key α_1 and α_2 isoform abundances underpin the upregulation in muscle NKA content with RT. In human skeletal muscle, the [^{3}H]ouabain binding assay can detect all of the α isoforms since each of α_1, α_2 and α_3 have high ouabain affinity (Wang et al. 2001). Hence the upregulation of muscle NKA content after RT may be due to increase in the abundance of both of the α_1 and α_2 isoforms, but possibly also the α_3 isoform, since this has the same ouabain affinity as the α_1 and α_2 in human skeletal muscle (Wang et al. 2001) However, this cannot be confirmed here since the α_3 isoform unfortunately could not be detected. Given that the NKA content is measured by the [^{3}H]ouabain binding site content technique, with high affinity binding of ouabain to both α_1 and α_2 isoforms, it was

important to assess if there were significant correlations between increased NKA content and increased NKA isoforms α_1 and α_2. The results demonstrate there was not a significant relationship between increased [^{3}H]ouabain binding site content and either of the α_1 and α_2 isoform abundances, although a tendency was noted for α_1 (P=0.077). This might likely reflect some large variations in α_1 and α_2 isoforms, making detection of significant correlations difficult.

The α_1 and α_2 isoforms are believed to play different physiological roles in muscle function (He et al. 2001; Lingrel et al. 2003; Radzyukevich et al. 2013). The NKA α_1 isoform is believed to be vital in Na^+ and K^+ exchange during basal conditions, whilst the α_2 isoform plays a key role during muscle contractions, thus improving exercise performance by enhancing fatigue resistance and maintaining muscle contractions (Radzyukevich et al. 2013). This suggests that the upregulation in both α_1 and α_2 isoform protein abundances with RT might differentially improve muscle performance, the increased α_1 isoform providing more rapid recovery, while the increased α_2 isoform may enhance contractions during exercise bouts.

Contrary to the study hypothesis, RT had no effect on the NKA β_1 isoform abundance, which also contrasts a previous report that the NKA β_1 abundance was increased after RT (Dela et al. 2004). The reason for the lack of change in the β_1 isoform with resistance training in this study is unknown. However, it is interesting that this is consistent with previous findings of unchanged β_1 isoform after each of intense intermittent exercise training (Nielsen et al. 2004), endurance training (Green et al. 2004) and acute high-intensity interval cycling training (Aughey et al. 2007). The lack of change in β_1 with RT may reflect an overall excess of β isoforms over α isoforms in muscle, which was suggested to be vital for NKA activity (Lavoie et al. 1997). Thus there is the possibility

that an existing overabundance of β isoforms may already be sufficient to form an increased number of functional NKA complexes and thus no further increase were induced during resistance training. However, this over abundances of β over α subunits is yet to be demonstrated in human skeletal muscle.

There was also no significant change in NKA β_2 isoform abundance after resistance training. Similar findings were reported following high-intensity interval cycling training in already well trained cyclists (Aughey et al. 2007) and endurance training in untrained participants (Benziane et al. 2011). In one study the β_2 isoform was shown to be increased with endurance training (Green et al. 2008). The reason for these discrepancies is unclear, and further study is needed to clarify the effects of training on the NKA β_2 isoform abundance.

This study sampled vastus lateralis muscle, which is of mixed fibre type composition (Staron et al. 2000). Further research is required to determine the effects of resistance training on NKA isoform abundances in single muscle fibres, to determine whether any fibre-type specific adaptations occur.

5.4.3 Conclusions

This study demonstrated that resistance exercise training induced an increase in muscle NKA content that was accompanied by an increases in abundance of both of the α_1 and α_2 NKA isoforms. These increases are important since the NKA is essential for muscle excitability. Thus upregulation of NKA α_1 and α_2 isoform$_s$ abundances may play key roles in regulating Na^+/K^+ exchange, membrane potential and excitability, both during and in recovery from resistance exercise.

Chapter 6. Effects of moderate and high intensity training on skeletal muscle NKA content and isoform abundances in patients with chronic kidney disease

6.1 Introduction

Skeletal muscle atrophy and a decline in muscle strength and function are common in patients with chronic kidney disease (CKD) (Domanski and Ciechanowski 2012; Workeneh and Mitch 2010). One factor affecting muscle function during strenuous exercise is muscle excitability (Sejersted and Sjøgaard 2000). A major function of K^+ in skeletal muscle is to polarise the muscle membrane, which is vital for muscle excitability and function (Nielsen and Clausen 2000). The fundamental role of the NKA α_2 isoform for survival is demonstrated in complete global knockout α_2 isoform mice, which are borne dead or die shortly after birth (Lingrel et al. 2003). Further, mice with a muscle-specific NKA α_2 isoform knockout exhibit substantially reduced muscle strength and enhanced muscle fatigue, suggesting that the α_2 is vital for muscle function (Radzyukevich et al. 2013). Partial global knockout of the α_1 isoform also decreased contractile force in mouse EDL muscle (Lingrel et al. 2003). The β isoform is believed to play a key role in regulation of NKA activity (Blanco and Mercer 1998; Hundal et al. 1994).

Patients with CKD have a lower (depolarised) resting membrane potential in skeletal muscle (Johansen et al. 2005; Cotton et al. 1979). Patients with CKD also demonstrated impaired plasma K^+ regulation during incremental cycling exercise, which was suggested to contribute to muscle fatigue and poor exercise performance (Petersen et al. 2011; Sangkabutra et al. 2003). In patients with CKD, endurance cycle training for six weeks improved exercise performance, despite unchanged K^+ regulation (Petersen et al. 2005). It has been suggested that impaired exercise performance in patients with CKD might be

due to depressed maximal muscle NKA activity (Petersen et al. 2005), yet no differences in muscle NKA content and isoform abundances were found compared to healthy participants (Petersen et al. 2011). The lack of difference in skeletal muscle NKA content and isoforms abundances was surprising and worthy of further investigation.

Regardless, an upregulation of the NKA content and isoform abundances in patients with CKD could be anticipated to potentially improve their muscle function and reduce fatigability. An upregulation of the muscle NKA content is associated with improve K^+ regulation which plays a critical role in preserving muscle excitability and preventing fatigue (McKenna et al., 2008). In healthy adults, the muscle NKA content was increased by 8 - 16% with each of high intensity interval, moderate intensity continuous, sprint, endurance and resistance exercise training (Wyckelsma et al. 2017; Green et al. 2008; Green et al. 2004; Evertsen et al. 1997) as also found with resistance training in this thesis (chapter 5). Importantly, no study has investigated the effects of exercise training on muscle NKA content and isoform abundances in CKD patients.

This study first compared muscle NKA content and α_1 - α_3 and β_1 - β_3 isoform abundances in CKD patients against healthy, age- and sex-matched participants. The effects of 12 weeks of moderate intensity continuous or high intensity interval exercise training on muscle NKA content and NKA α_1 - α_3 and β_1 - β_3 isoform abundances in patients with CKD were then investigated. The hypotheses tested were first that there would be no differences in muscle NKA content and isoform abundances between CKD patients and healthy participants, and second, that the muscle NKA content and the NKA α_1, α_2, and β_1 isoforms abundances would each be increased following both moderate and high intensity running exercise training.

6.2 Methods

6.2.1 Participants and overview

Fifteen patients with stage 3-4 CKD, with estimated Glomerular Filtration rate (eGFR) of 29-60 ml/min/1.73m^2, comprising six females and nine males, were recruited for this study. Patients underwent either moderate intensity continuous training (MICT, n = 5), or high intensity interval training (HIIT, n = 8), whilst 2 patients with CKD acted as controls (CON-CKD). Fifteen healthy age- and sex-matched control participants act as control (CON). The physical characteristics for the CKD patients were; age: 61.9 ± 7.9 yr; height: 165.0 ± 8.0 cm; body mass: 83.76 ± 17.23 kg, whilst for the healthy controls were age: 62.8 ± 8.3 yr; height: 173.7 ± 7.9 cm; body mass: 95.2 ± 16.2 kg (mean±SD).

The self-reported active questionnaire was used to evaluate average weekly physical activity levels. Total physical activity was calculated from walking, moderate intensity and vigorous intensity time in an average week (eg. moderate = gentle swimming/social tennis/golf; vigorous = jogging/cycling/aerobics/competitive tennis). The HIIT group reported performing higher levels of physical activity (3.5 hours) per week compared to the MICT group (1.5 hours) (Beetham 2015). At the beginning of the study, twelve participants were randomised into the HIIT group and ten to the MICT group. After eight withdrawals, there were nine participants who completed the HIIT intervention and five who completed the MICT intervention. Due to the difficulty of recruiting patients with CKD, and since five participants withdrew from the study only two participants completed the study as CKD control group.

None of the patients with CKD were on dialysis treatment, but they were on the following medications: ace-inhibitor n = 9, β-blocker n = 4, thiazide n = 2, statin n = 8 and allopurinol n = 4. All participants gave written, informed consent to participate in the study. They

were then familiarised with training and undertook an incremental exercise test to determine VO_{2peak} with measurement of maximal heart rate (HR_{max}) to determine the heart rate target for training intensities. On a separate day, participants underwent a resting vastus lateralis muscle biopsy prior to training; a post-training biopsy was taken at rest, 48 - 72 h after the final training session.

This study was part of a larger collaborative project with Associate professor Jeff Coombes and Dr Kassia Beetham, School of Human Movement Studies, the University of Queensland. All protocols and procedures for CKD patients including testing, collection of muscles samples and training of participants were undertaken by these investigators at the St Lucia campus, The University of Queensland. All protocols and procedures were approved by the University of Queensland Human Research Ethics Committee, and the Princess Alexandra Hospital Human Research Ethics Committee. Participants for the healthy control group were recruited and muscle tissues collected and analysed at Victoria University, and all procedures were approved by the Victoria University Human Research Ethics Committee.

6.2.2 Training Program

The training protocol comprised three training sessions per week over 12 weeks with training conducted on a motorised treadmill (Quinton Q65, Seattle, WA, USA). Exercise intensities for the training were based on the HR_{max}. Both HIIT and MICT groups performed a warm-up at 50 - 60% HR_{max} for 5 min and following each training session, both groups performed a three minute cool down at 50-60% HR_{max}. The HIIT comprised 4 intervals of 4 min duration at 85-95% HR_{max}, with an intervening 3 min of active recovery at 65% HR_{max}; each training session lasted for ~33 min. The MICT comprised 30 min exercise bouts at 65% HR_{max} and each training session was for ~40 min duration. Progressive overload was applied with training via increasing the speed of the treadmill

as required to ensure that participants reached HR target. The heart rate was monitored during the training using a heart rate monitor (RS800sd, Poar Elctro Oy, Kempele, Finland). Compliance with the training program was 94 ± 2%. Exercise training was undertaken at the University of Queensland, The Princess Alexandra Hospital, Logan Hospital and Browns Plains Community Centre, Brisbane, Australia and was conducted under supervision of an Accredited Exercise Physiologist.

6.2.3 Muscle samples.

The muscle biopsy procedure was identical to that described in Chapter 4.

6.2.4 [^{3}H]-ouabain binding site content

Analysis of muscle NKA content was determined by the [^{3}H]-ouabain binding site content assay performed, as described in Chapter 4.

6.2.5 Western blotting

NKA isoform abundances (α_1 - α_3, β_1 - β_3) were measured using the same western blotting techniques as described in Chapter 3.

6.2.6 Statistics

An independent Student t-test was used to determine differences at baseline between CKD patients and the healthy controls. The effects of training on muscle [^{3}H]ouabain binding site content and NKA isoform abundances were evaluated within each group via a one-tailed paired t-test (pre vs post) with training since training was predicted to increase these measures. No statistical analysis was conducted on the CKD control group, pre vs post training (CKD-CON, n = 2). Data are presented as mean ± standard deviation (SD) and statistical significance was set at $P < 0.05$. Statistical analyses were conducted using SPSS version 24. Effect size was calculated using Cohen's *d*, where <0.2, 0.2-0.5

and 0.5-0.8 and >0.8 are considered trivial, small, moderate and large, respectively (Cohen 1988). All figures were created using SigmaPlot 13 (Systat Software, Inc).

6.3 Results

6.3.1 Comparisons between patients with CKD and healthy controls

6.3.1.1 Muscle NKA content

The muscle NKA content did not differ between CKD at baseline and healthy controls (CON) ($p = 0.459$, $d = 0.228$, Fig 6.1).

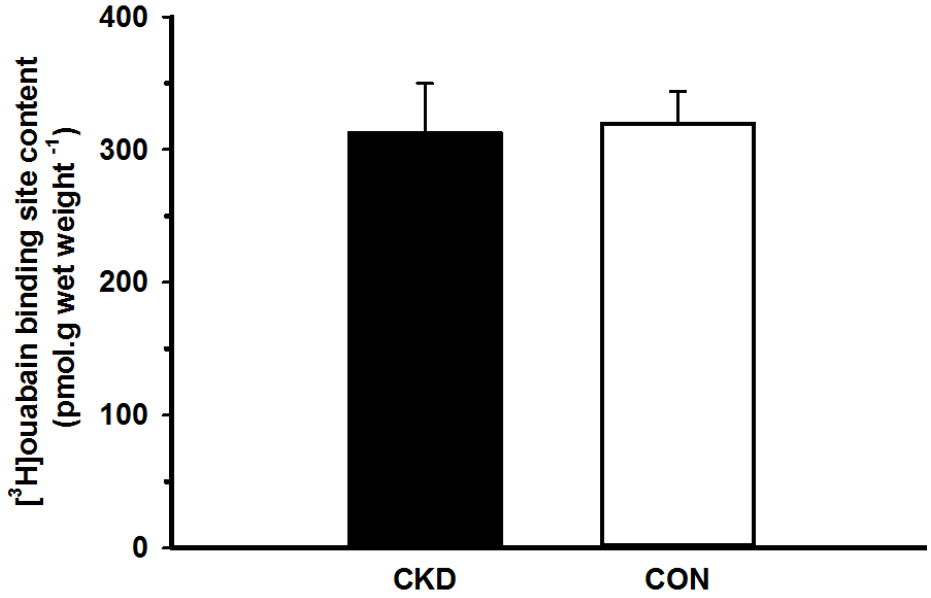

Figure 6.1. Comparison of skeletal muscle NKA content between between patients with CKD and healthy controls.

Data are presented as mean ± SD in pmol.g wet.weight^{-1}, n = 15 per group.

6.3.1.2 Muscle NKA isoform abundances

Representative blots for NKA α_1, α_2, β_1 and β_2 are shown in Fig 6.2. Neither the NKA α_3 nor the β_3 isoforms could be detected, despite attempts at several total protein mounts and using two different antibodies.

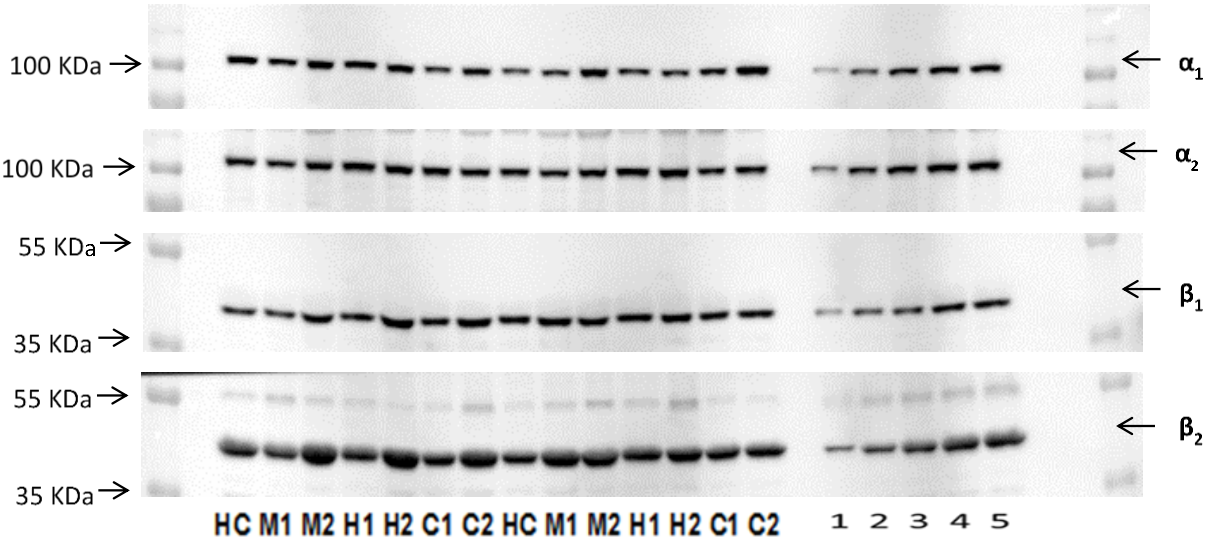

Figure 6.2. Representative immunoblots of NKA α_1, α_2, β_1 and β_2 isoforms in homogenates of human vastus lateralis muscle.

Values at left indicate molecular weight of bands. Protein bands from left to right are (HC) healthy control; (M1) pre-training moderate intensity continuous training; (M2) post-training moderate intensity continuous training; (H1) pre-training high intensity interval training, (H2) post-training high intensity interval training; (C1) pre- control CKD; (C2) CKD post control. 1, 2, 3 and 4 are calibration curve loaded with 2.5 - 12.5µg whole-muscle crude homogenate. The homogenate was prepared from an equal amount from each sample

6.3.1.2.1 Muscle NKA α isoform abundance

There were no differences in NKA α_1 ($p = 0.984$) or α_2 ($p = 0.235$, $d = 0.538$) isoform abundances between CKD and healthy controls (CON) (Fig 6.3).

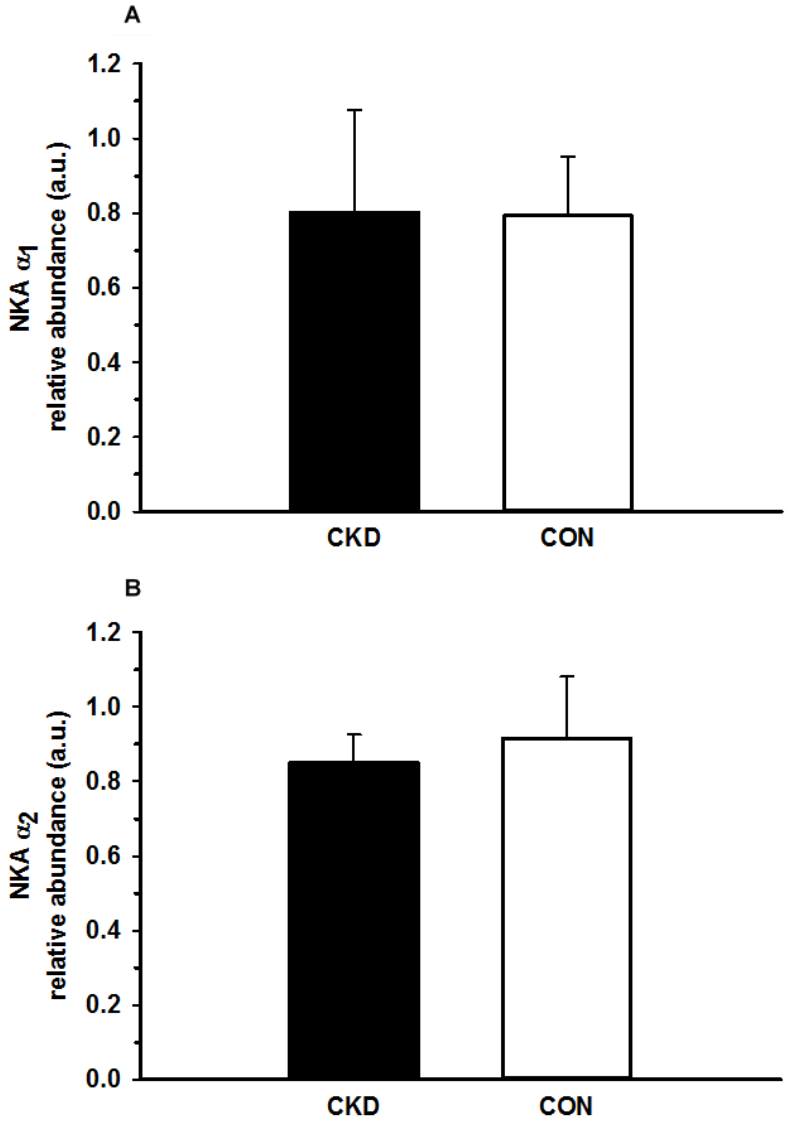

Figure 6.3. Comparison of skeletal muscle NKA (A) α_1 and (B) α_2 isoform aboundances between CKD patients and healthy controls.

Data are presented as mean ± SD in arbitrary units (a.u.), n = 15 per group.

6.3.1.2.2 Muscle NKA β isoform abundance

There were also no differences in NKA β_1 (p = 0.247, d = 0.437) or β_2 (p = 0.138, d = 0.451) isoform abundances between CKD and healthy controls (CON) (Fig 6.4).

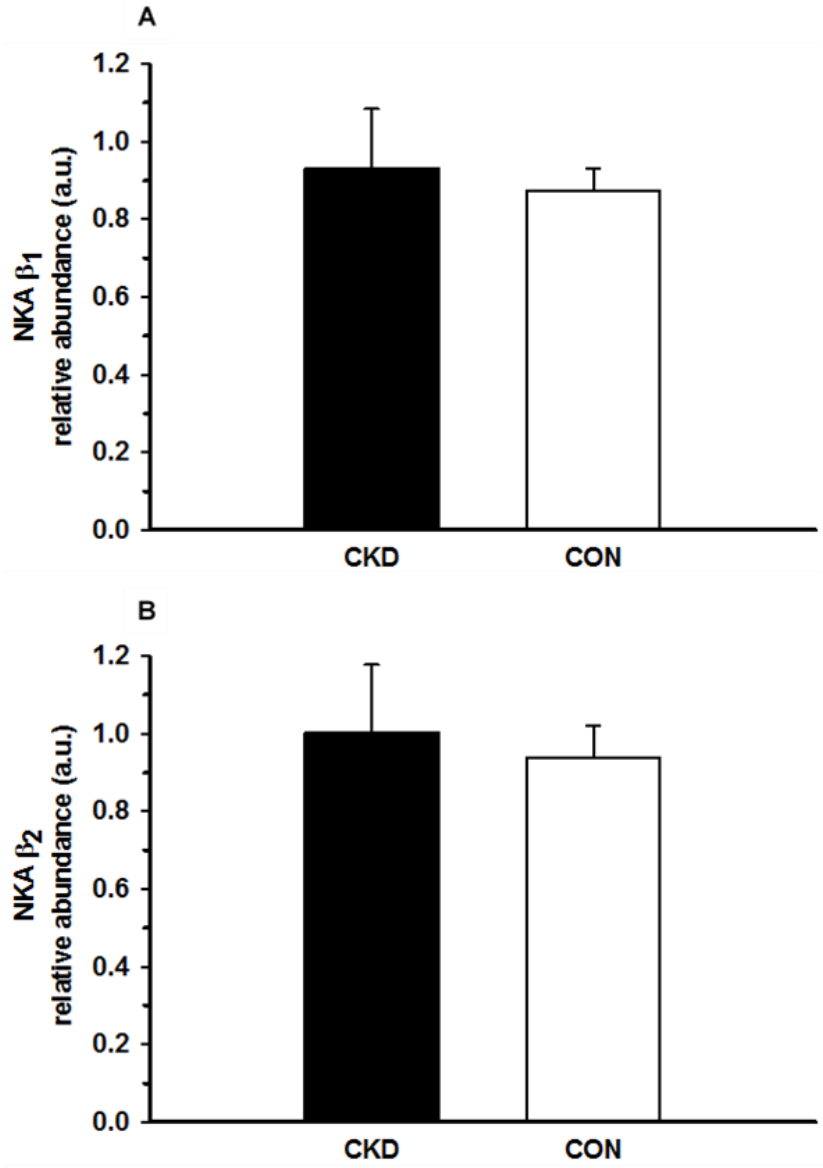

Figure 6.4. Comparison of skeletal muscle NKA (A) β_1 and (B) β_2 isoform aboundances between CKD patients and healthy controls.

Data are presented as mean ± SD in arbitrary units (a.u.), n = 15 per group.

6.3.2 Effects of moderate and high intensity training on muscle NKA content and NKA isoform abundances

6.3.2.1 Muscle NKA content

No significant differences in muscle NKA content were found following MICT ($p = 0.165$, $d = 0.375$) or HIIT ($p = 0.278$, $d = 0.237$, Fig 6.5); there also appeared to be no change in CKD-CON (n=2, Fig 6.5). Pooled muscle from MICT and HIIT before training did not differ from following training (n = 13, 309.5 ± 25.4 vs 307.2 ± 9.9 pmol.g wet.weight^{-1}, $p = 0.110$, $d = 0.117$).

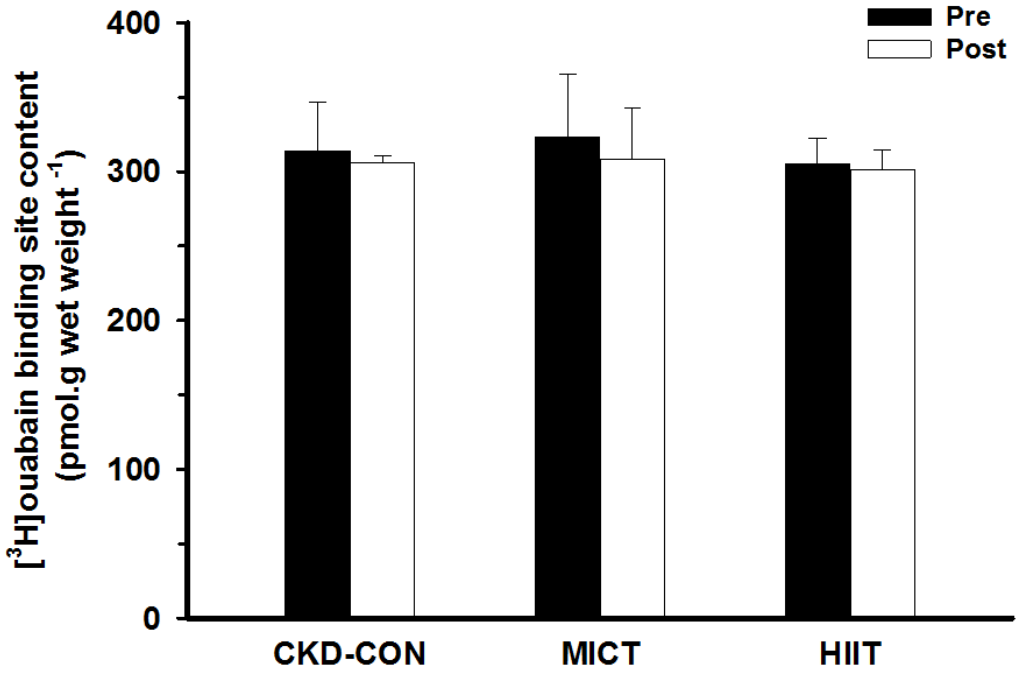

Figure 6.5. Skeletal muscle NKA content before and following 12 wk of moderate intensity continuous (MICT), or high intensity interval training (HIIT).

Data are presented as mean ± SD in arbitrary units (a.u.), n = 5 MICT, n = 8 HIIT and n = 2 CKD-CON.

6.3.2.2.1 Muscle NKA α isoform abundances

There were no differences in NKA α_1 or α_2 isoform abundances following MICT ($p = 0.420$, $d = 0.145$; and $p = 0.086$, $d = 1.42$, respectively), or HIIT ($p = 0.389$, $d = 0.166$ and $p = 0.275$, $d = 0.441$, respectively, Fig 6.6); there also appeared to be no change in CKD-CON (n=2, Fig 6.6). However, when data was pooled from MICT and HIIT groups, the NKA α_2 isoform was increased after training by 8% (n = 13, 0.87 ± 0.12 vs 0.94 ± 0.10 a.u., $p = 0.035$, $d = 0.633$); no differences were found in pooled α_1 after training ($p = 0.360$, $d = 0.608$).

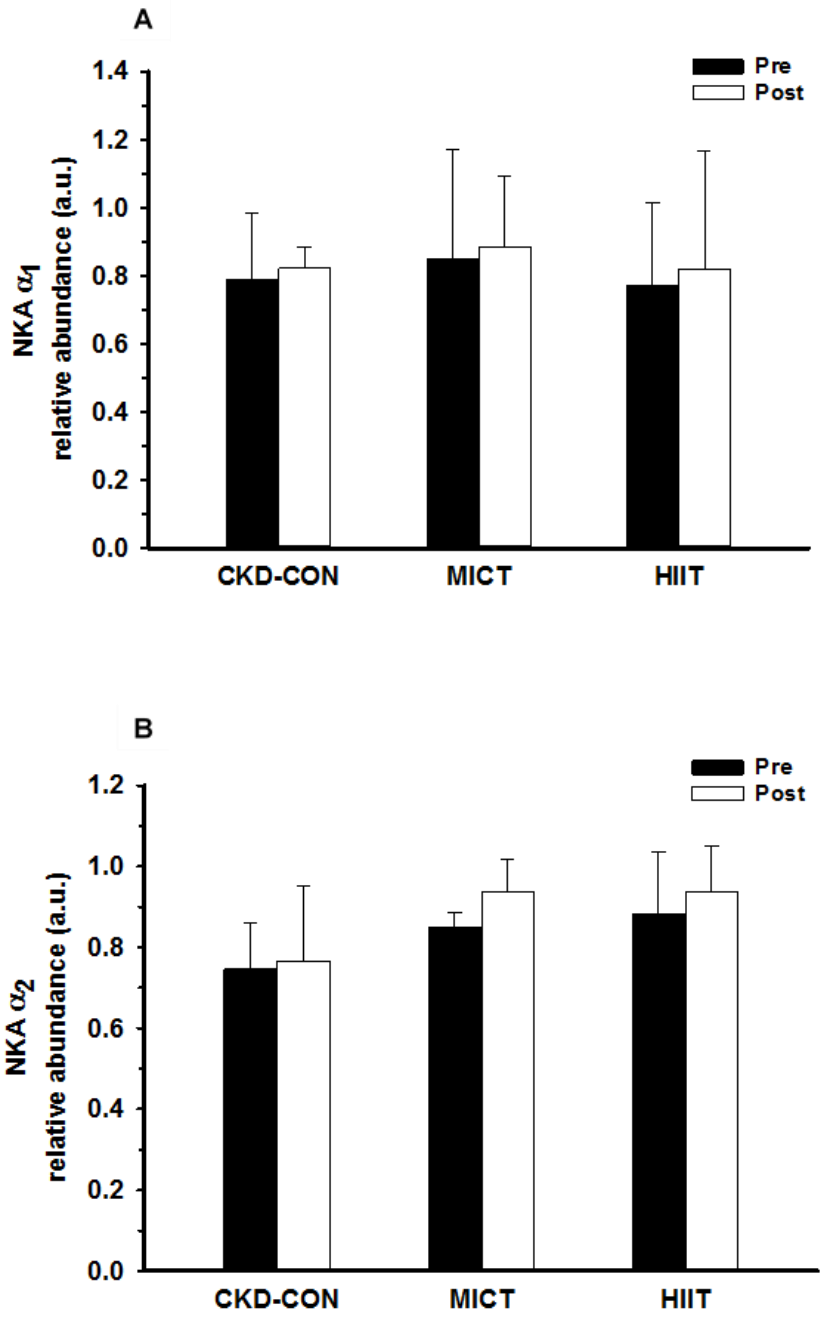

Figure 6.6. Skeletal muscle NKA (A) α_1 and (B) α_2 isoform abundances before and following 12 wk of moderate intensity continuous, (MICT) or hgh intensity interval training (HIIT).

Data are presented as mean ± SD in arbitrary units (a.u.), n = 5 MICT, n = 8 HIIT and n = 2 CKD-CON.

6.3.2.2.2 Muscle NKA β isoform abundances

There were no differences in NKA β_1 or β_2 isoform abundances following MICT ($p = 0.340$, $d = 0.114$ and $p = 0.158$, $d = -0.411$, respectively), or HIIT ($p = 0.237$, $d = 0.099$, $p = 0.213$, $d = 0.242$, respectively, Fig 6.7); there also appeared to be no change in CKD-CON (n=2, Fig 6.7). Pooled data from MICT and HIIT also revealed no significant differences in NKA β_1 or β_2 isoform abundances after training ($n = 13$, $p = 0.379$, $d = 0.108$, and $p = 0.262$, $d = 0.312$, respectively).

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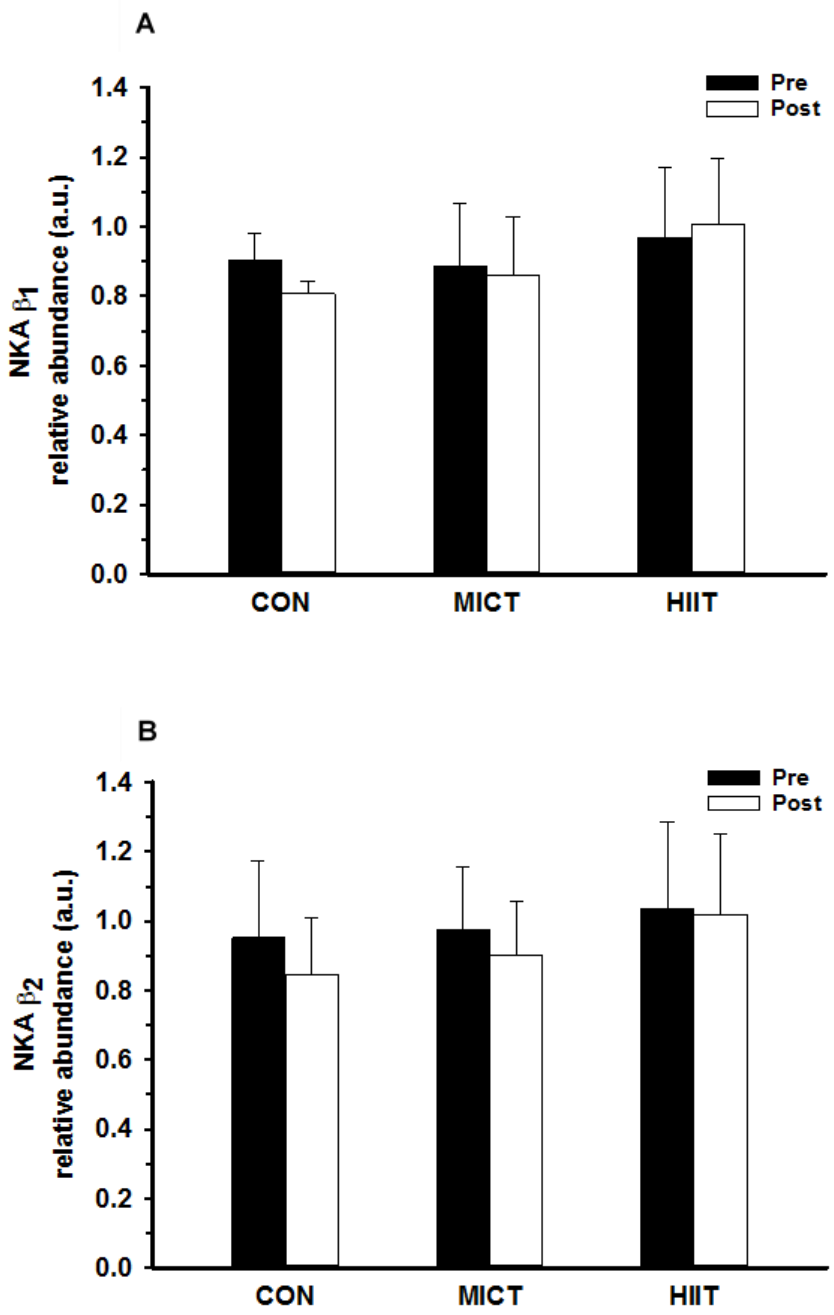

Figure 6.7. Skeletal muscle NKA (A) β_1 and (B) β_2 isoform abundances before and following 12 wk of moderate intensity continuous, or high intensity interval training

Data are presented as mean ± SD in arbitrary units (a.u.), n = 5 MICT, n = 8 HIIT, n = 2 CKD-CON

6.4 Discussion

This study firstly found no differences in skeletal muscle NKA content and NKA α_1 - α_2 and β_1 - β_2 isoform protein abundances between patients with CKD and age- and sex-matched healthy controls. The study then investigated the effects of 12 weeks of moderate intensity continuous (MICT) and high intensity interval training (HIIT) on muscle NKA content and NKA isoform abundances in patients with CKD. Contrary to the hypothesis, exercise training did not increase any of the skeletal muscle NKA content, or NKA α_1, α_2, β_1 and β_2 isoform abundances in the participant with CKD. However, when both MICT and HIIT data were pooled the NKA α_2 was increased by 8% after training, with no changes in other NKA measures.

6.4.1 NKA content and NKA isoform abundances in patients with CKD vs healthy people

No differences in NKA content and α_1, α_2, β_1 and β_2 isoform abundances were found between patients with CKD and healthy controls, confirming the previous finding by Petersen (2011). The previous study reported high variability in western blot results for CKD and a smaller sample size, thus these finding extend this early study, confirming these results in a larger sample, with less variability. Thus, it can be concluded that there were no differences in muscle NKA content and NKA isoform protein abundances between patients with CKD and healthy controls. This finding is consistent with an earlier study in rat muscle (Druml et al. 1988). This suggests that the lower NKA activity in CKD reported earlier (Petersen et al. 2011) was unlikely to be due to decreased NKA content or NKA isoform abundances, but may reflect alterations in other regulators such as FXYD1, or some inhibition of activity per se.

6.4.2 Effects of moderate intensity continuous and high intensity interval exercise training on muscle NKA content and isoforms

The skeletal muscle NKA content did not increase after 12 weeks of MICT or HIIT, or even with data pooled together. This finding was unexpected, given that exercise training commonly induces a marked upregulation in muscle NKA content in healthy individuals (section 2.7.2) and also in patients with type 1 diabetes (Harmer et al. 2006). In this study, neither form of training increased any of the NKA α_1, α_2, β_1 or β_2 isoform abundances. Given the inconsistencies in the literature regarding the response of NKA isoforms abundances to exercise training (Table 2.1, chapter 2), these findings are not surprising, but are demonstrated, here for the first time with training in CKD. In human muscle, the NKA content is measured by the [^{3}H]ouabain binding site content technique, with high affinity binding of ouabain to both α_1 and α_2 isoforms. Hence the finding of unchanged α_1 and α_2 isoforms is consistent with the unchanged [^{3}H]ouabain binding site content with each training regime.

One possible explanation for the lack of training effect on NKA content could be due to a likely smaller muscle fibre size in patients with CKD. It has been suggested there is a link between NKA content and muscle fibre size, with [^{3}H]ouabain binding site content measures being associated with muscle fibre plasma membrane area (Harrison et al. 1994). Muscle atrophy is common and progressive in patients with CKD (Johansen et al. 2003; Zhang et al. 2011), so they also exhibit smaller muscle fibre size (Sakkas et al. 2003). Therefore these patients with CKD most likely would have a greater sarcolemma density, which if correct, would indicate a less NKA content for a given area of sarcolemma. If so, this might be detected also in CKD vs CON but this was not the case.

Each of these variables need to be measured to determine whether such differences might exist.

The lack of increase in muscle NKA content and isoforms after training in patients with CKD might alternately be due to the training protocols employed providing an insufficient stimulus to upregulate NKA. The lack of upregulation NKA content in this study was consistent with no associated improvement in VO_{2peak} with both MICT (1.80 ± 0.04 vs 1.82 ± 0.34 $L.min^{-1}$) and HIIT (1.97 ± 0.06 vs 2.00 ± 0.63 $L.min^{-1}$) training protocols (Beetham 2015). For study 4 CKD performance results see appendix 7.

This raises the possibility that patients did not train for sufficiently long duration or at a high enough intensity to evoke an increased NKA content. However, this seems unlikely since most training programs lasting for 12 weeks or shorter duration, even with lower exercise intensity increased muscle NKA content (Table 2.4).

Due to the difficulty of recruiting patients with CKD to undertake prolonged training, a relatively small sample size was used. Thus it could also be that the lack of substantiated training effects on NKA content and NKA isoform abundances here may reflect a type II error. With a pooled sample size of 13 participants and low variability for NKA content, it would be anticipated to detect an increase in NKA content. A small effect size for NKA content in MICT (d = 0.353) and in HIIT (d = 0.237), might suggest a slight change in NKA content with training. However, if anything this would have been a reduction not an increase as expected. Furthermore, this seems unlikely since a trivial effect size was found in the pooled data (p = 0.117). Pooled data from MICT (n = 5) and HIIT (n = 8) did however show a significant increase in NKA α_2 isoform after training, consistent with a possible type II error for this measure. Further studies with a larger sample size are needed to confirm the effect of the specific training program on α_2 isoforms. It is however

difficult to explain without additional and/or mechanistic information as to why no increase was found' It is unclear why the discrepancy existed between increased pooled α_2 abundance and unchanged pooled NKA content. This suggests than an increased abundance of α_2 isoforms were not associated with a corresponding increase in $\alpha_2\beta$ complexes. This could be suggest an important role in forming functional NKA and thus might be consistent with an earlier report of increase activity patients with CKD. Another possible explanation for the lack of training effect on NKA content could be that CKD may have blocked biochemical signalling pathways of the non-ion transducing role of NKA at least for α_1 isoform, thus further research on the effect of CKD on NKA biochemical signalling pathways is needed.

It should be noted that patients with CKD in this study typically took multiple medications including ace-inhibitors, β-blockers, thiazide, statins and allopurinol. It is unclear whether any of these medications may impact directly to block adaptability of muscle NKA with training.

Endogenous ouabain acts as an inhibitor of the NKA (Paczula et al. 2016). Given that CKD patients have high levels of endogenous ouabain (Stella et al. 2008), which led to inhibited NKA activity in erythrocytes by 56% compared to control (Kolmakova et al. 2011), it is possible that elevated endogenous ouabain blocked the upregulation of muscle NKA with training.

6.4.3 Conclusions

This study demonstrates that CKD had no effect on muscle NKA content or NKA isoform protein abundances compared to healthy controls. A surprising finding was that MICT and HIIT each failed to induce an upregulation in NKA content and isoform abundances in patients with CKD. An exception to this was the upregulation in α_2 isoform when data

was pooled from both MICT and HIIT. Further research is required to ascertain the mechanisms explaining the lack of adaptability in muscle in CKD. Further research might also include with different training protocols such as resistance training (Chapter 5) to see whether adaptability to this training stimulus is also blocked.

Chapter 7. General discussion, conclusions and recommendations for future research

Findings of this thesis have already been discussed in detail in each respective chapter. This chapter therefore provides only high-level summary, and then integrates these finding where possible, making further relevant comments conclusions, and recommendations for further research.

7.1 General discussion

7.1.1 Immobilisation, testosterone and recovery in rat skeletal muscle

A novel aspect of this thesis was investigation of the effects of castration, which resulted in 90% reduction in testosterone, on skeletal muscle NKA regulation measured via [^{3}H]ouabain binding site content and NKA isoform abundances in rat soleus muscle (Chapter 3). A major finding was that castration impaired the recovery of muscle NKA content and α_2 abundance after their reduction caused by hindlimb immobilisation. The [^{3}H]ouabain binding site content was decreased with immobilisation in both sham and castration groups by 34%. In the sham group, the [^{3}H]ouabain binding site content had recovered after 14 days, which was consistent with previously reported recovery after 7 days to 5 weeks (Zemkova et al. 1990; Leivseth et al. 1992). However, in the castration group, the [^{3}H]ouabain binding site content remained depressed by 34% after 14 days of recovery. This suggests an important role for testosterone in muscle NKA recovery from sub-normal levels. The α_2 is the dominant NKA α subunit in rat soleus muscle representing ~80 - 85% of α subunits (He et al. 2001; Hansen and Clausen 1988). The downregulation of the [^{3}H]ouabain binding site content in the castration group coincided with reductions in the α_2 isoform, following immobilisation and after 14 days of recovery. However, in the sham group the α_2 isoform abundance was unchanged with

immobilisation despite the reduction of [^{3}H]ouabain binding site content. It is unclear why this discrepancy existed. An important finding was that abundances of the NKA β_1 and β_2 isoforms were unaffected by hindlimb immobilisation or castration alone, however, clear effects were found when the hindlimb immobilisation was combined with castration. Thus testosterone suppression via castration reduced the NKA β_1 and β_2 isoform abundances after immobilisation. This suggests that combined hindlimb immobilisation with castration caused a reduction in both NKA $\alpha_2\beta_1$ and $\alpha_2\beta_2$ complexes.

7.1.2 Training adaptation in human skeletal muscle

Studies 2 - 4 investigated NKA adaptability in human skeletal muscle with physical training. In study 3, resistance training for 8 weeks led to increased NKA content and elevations in both of the main NKA α isoforms, α_1 and α_2 (Chapter 5). This contrasted with study 2 where none of the NKA content or α isoform abundances were significantly changed after 7 weeks of sprint training (Chapter 4). The lack of adaptation in muscle NKA content after sprint training was unexpected, considering that two previous studies reported an increase in NKA content with an identical training protocol (McKenna et al. 1993; Harmer et al. 2006). The reasons for this are not clear, however, six of the eight participants in that study did respond to training with an increased NKA content (46.8%, $p = 0.017$). This strongly suggests that the sprint training regime was capable of increasing NKA content. This percentage increase was however, considerably higher than previous studies that reported 8 - 16% mean increases with the same training protocol (McKenna et al. 1993; Harmer et al. 2006). Overall the unchanged NKA content was not accompanied by any changes in the NKA α isoform abundances.

Study 4 (Chapter 6) firstly confirmed a previous finding of no difference in skeletal muscle NKA content and NKA isoform abundances between patients with CKD and

healthy individuals (Petersen et al 2011). However, in that study patients with CKD were on haemodialysis and renal transplant recipient treatments, whilst in this thesis none of the patients with CKD were on dialysis or transplant recipient treatments. Thus these findings extend previous knowledge and suggest that CKD has no effect on muscle NKA despite in which stage of the patients with CKD in.

A major finding was that neither moderate intensity continuous (MICT) or high intensity interval training (HIIT) induced an upregulation in muscle NKA content or NKA isoform abundances in these CKD patients. This finding contrasts with numerous previous studies investigating training in healthy individuals, which reported upregulation of NKA content and of NKA isoform abundances (see Table 2.4). When data from MICT and HIIT were pooled, however, the NKA α_2 isoform was significantly increased after training by 8%, although NKA content and α_1 isoform were still unchanged. These combined results show for the first time that NKA α_2 isoform abundance can be increased with training in patients with CKD.

In rat soleus muscle, NKA α_1 isoform has a lower affinity for [^{3}H]ouabain, thus the [^{3}H]ouabain site content assay utilised in this thesis does not detect α_1 subunits (Clausen 2003; Hansen 2001); the α_1 isoform represents around ~20% of the NKA, whilst the α_2 isoform comprised ~80 - 85% of the NKA (Hansen 2001; He et al. 2001; Hansen and Clausen 1988). Hence the decreased [^{3}H]ouabain site content in rat soleus muscle in study 1 (Chapter 3) is due to decreased α_2 content, although could also include some contribution from the decrease in α_3 isoform. However, in human skeletal muscle, the NKA α_1, α_2, and α_3 isoforms each have a similar ouabain affinity (Wang et al. 2001), thus the increased [^{3}H]ouabain site content in study 2 (Chapter 4) and 3 (Chapter 5) reflects the whole NKA content and the sum of all changes in α isoforms.

The results from study 1 (Chapter 3) indicates that the muscle NKA content is subject to downregulation, caused by immobilisation and accompanied immobilisation with castration and study 3 (Chapter 5) indicates upregulation with resistance training. The NKA content is vital at rest and during muscle contraction for Na^+/K^+ exchange and Em regulation(Ewart and Klip 1995; Clausen 2003b). A reduction of [^{3}H]ouabain binding site content might reduce the capability of skeletal muscle to maintain excitability and contribute to muscle fatigue, which may lead to reduced muscle function (McKenna et al. 2008). Hence restoration of the capacity of NKA after immobilisation is likely to be important for regaining muscle function. During intense muscle contractions there is an urgent needed for clearance of extracellular K^+ to prevent muscle fatigue (Clausen 2013b; Nielsen and Clausen 2000), thus a high capacity of NKA is required to meet this demand. This thesis shows that resistance training in healthy young adults is effective at increasing the capacity of NKA via upregulation of the NKA content. Thus, it is interesting to speculate that resistance training may be beneficial to restore muscle [^{3}H]ouabain site content in participants where this has been reduced after immobilisation, or in patients with chronic disease that also are affected by chronic inactivity.

Some key findings of this thesis are summarised graphically in Figures 7.1 and 7.2, which respectively present the percentage changes in [^{3}H]ouabain binding site content and isoform relative abundances from all studies, including with humans and rats. This demonstrates firstly there was not a consistent relationship between adaptability of [^{3}H]ouabain binding site content and either of the α_1 and α_2 isoform abundances. Secondly, it demonstrates some large variations in percent changes, particularly of α_1 isoform, making detection of changes difficult. Thirdly, it demonstrates that a larger change in β_1 and β_2 isoform abundances with castration in the rat study, but only small

changes that indicate an overall lack of β isoforms adaptability in human of skeletal muscle.

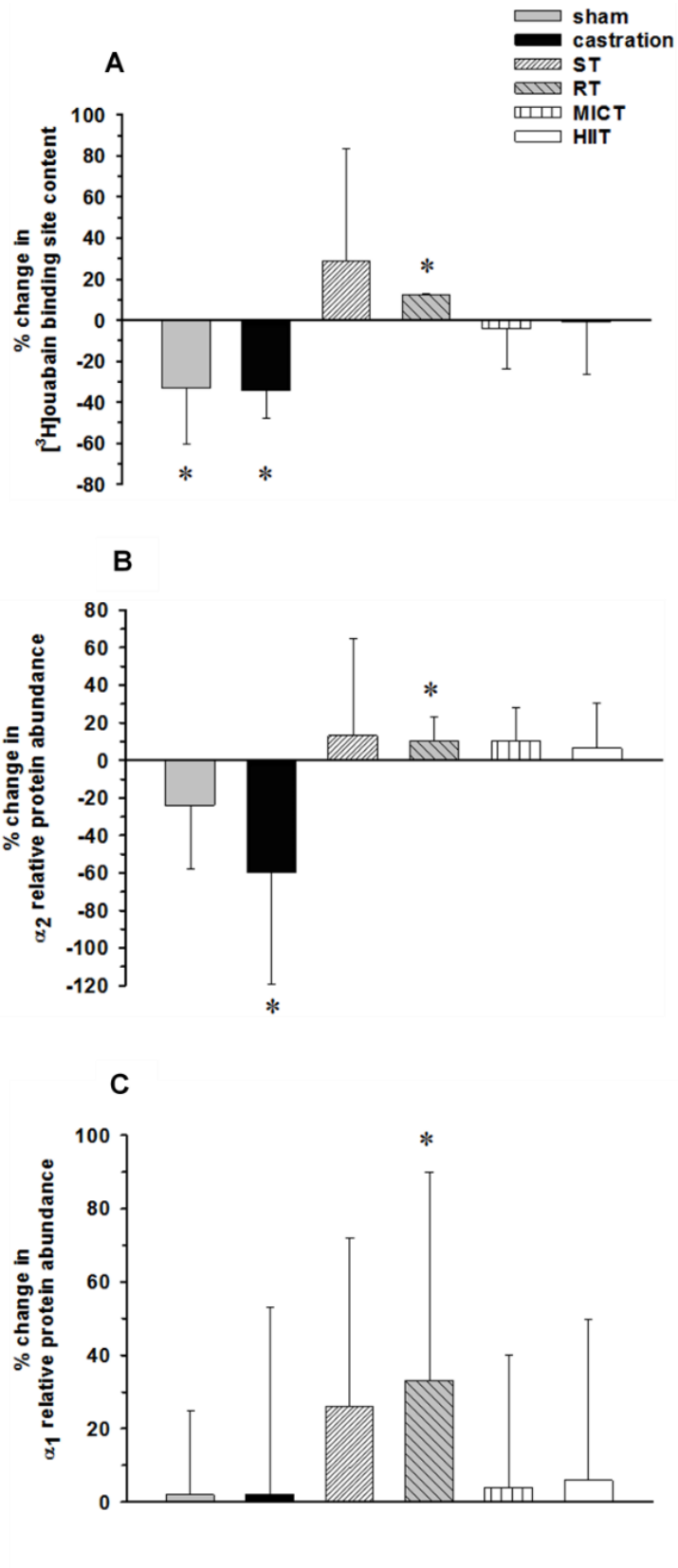
sham
castration
ST
RT
MICT
HIIT
A
% change in [3H]ouabain binding site content
100
80
60
40
20
0
-20
-40
-60
-80
*
*
*
B
% change in α2 relative protein abundance
80
60
40
20
0
-20
-40
-60
-80
-100
-120
*
*
C
% change in α1 relative protein abundance
100
80
60
40
20
0
*

Figure 7.1. Percentage changes in skeletal muscle [^{3}H]ouabain binding site content (A) and α_2 isoform (B) α_1 isoform (C) in rats after hindlimb immobilisation in sham and castration groups, in healthy human adults after resistance training (RT), sprint training (ST) and in CKD patients after moderate intensity continuous (MICT) and high intensity interval training (HIIT). Results from control groups are not included.

Error bars represent SD, * significantly different, $p < 0.05$ from control in rat study, and post-training from pre-training in human studies.

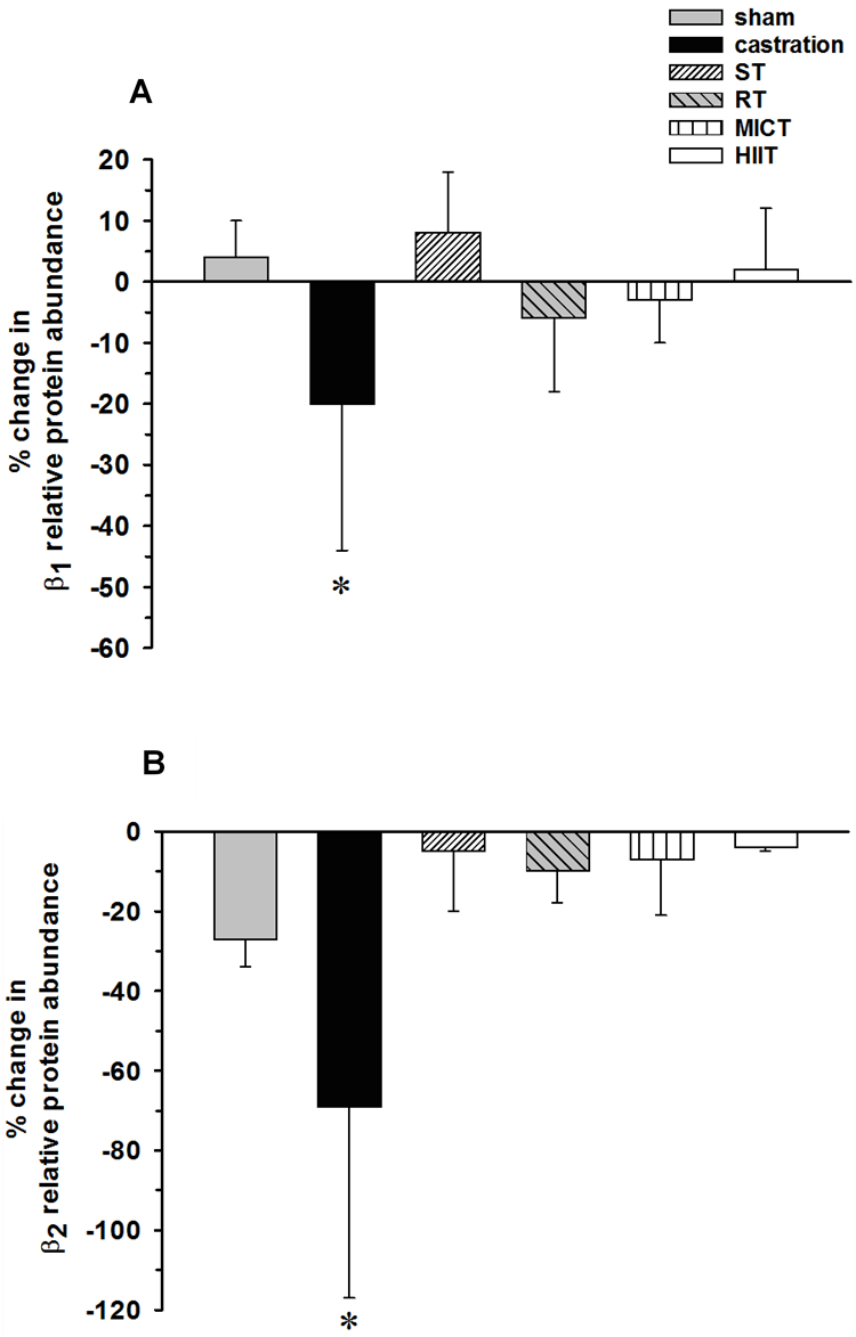

Figure 7.2. Percentage change in skeletal muscle NKA β_1 isoform (A) and β_2, isoform (B) in rats after hindlimb immobilisation in sham and castration groups, in healthy human adults and after resistance training (RT), sprint training (ST) and in CKD patients after moderate intensity continuous (MICT) and high intensity interval training (HIIT). α_3 isoform only detected in rat muscle and not presented here, Resulsts from control groups not included.

Error bars represent SD, * significantly different from control in rats study, and from pre-training in human studies.

7.2 Conclusions

The major conclusions from this thesis are:

Study 1, Chapter 3. Effects of hind limb immobilisation and castration on [^{3}H]ouabain binding site content and NKA isoform abundance in rat soleus muscle

1. Ten days of hindlimb immobilisation reduced muscle [^{3}H]ouabain binding site content in both the sham and castration groups by 34%.
2. After 14 days of recovery, muscle [^{3}H]ouabain binding site content was restored to control levels in the sham group, but remained depressed in the castration group by 34%.
3. Following 10 days of hindlimb immobilisation, none of the NKA α_2, β_1 and β_2 isoform abundances were changed in the sham group, but each were downregulated in the castration group by 60, 26, 65%, respectively, and also castration blocked the α_2 isoform recovery and remained depressed by 42%.
4. Ten days of hindlimb immobilisation caused no changes in the NKA α_1 or α_3 isoform abundances in both sham and castration groups.

Study 2, Chapter 4. Effects of sprint training on skeletal muscle NKA content and isoform abundance in human skeletal muscle.

Following 7 weeks sprint training:

1. The muscle NKA content was not significantly increased, but tended to increase, being significantly elevated in six of the 8 participants by 46.8%.
2. None of the NKA α_1, α_2, β_1 and β_2 isoform abundances were significantly increased.

Study 3, Chapter 5. Effects of resistances training on skeletal muscle NKA content and isoform abundance in human skeletal muscle.

Following 7 weeks of resistance training:

1. The muscle NKA content increased by 12%.
2. Both of the NKA α_1 and α_2 isoform abundances increased 32, 10%, respectively, but the β_1 and β_2 isoform abundances were unchanged.

Study 4, Chapter 6. Effects of moderate and high intensity training on skeletal muscle NKA content and isoform abundances in patients with chronic kidney disease

1. No differences were found between patients with CKD and healthy controls in muscle NKA content or in NKA α_1, α_2, β_1, and β_2 isoform abundances.
2. Moderate intensity continuous and high intensity interval training did not change any of muscle NKA content and NKA α_1, α_2, β_1 or β_2 isoforms abundance in patients with CKD.
3. In data pooled from both groups after training the α_2 isoform was increased by 8%.

7.3 Recommendations for future research

Further research would benefit by also investigating the effects of hindlimb immobilisation and castration on skeletal muscle [^{3}H]ouabain binding site content and NKA isoforms abundances, in muscle rich in fast twitch fibres, such as extensor digitorum longus (EDL) or the white portion of the gastrocnemius muscle. This would extend these finding on soleus muscle. Further research should investigate possible underlining biochemical pathways involved by which testosterone suppression via castration blocked the recovery of [^{3}H]ouabain binding site content after hindlimb immobilisation in rat muscle. Furthermore, research should explore whether similar effects of testosterone suppression occur in skeletal muscle NKA in humans especially in men patients with prostate cancer, type 2 diabetes or elderly.

Estradiol hormone has shown to stimulate the NKA activity in cardiac muscle in rats (Džurba et al. 1997; Obradovic et al. 2015; Obradovic et al. 2015). However, no studies have investigated effects of estradiol on NKA in skeletal muscle. Further research should therefore investigate the effects of estradiol on skeletal muscle NKA regulation.

Further research employing a large sample size in each different exercise protocols are needed to verify the lack of change in muscle NKA content and NKA isoform abundances with moderate intensity continuous and high intensity interval training in patients with CKD. This lack of change might be due to the smaller sample size as emphasised by pooling data from MICT and HIIT, which showed an NKA α_1 isoform after training. Furthermore, research should explore whether intramuscular signalling pathway involved with α_2 isoform may be linked to this lack of adaptation in NKA, and whether that specific training regime would increase skeletal muscle NKA content and isoform abundances in patients with CKD. It is that possible differences between patients with CKD and healthy

individuals may be at least be partially due to different physical activity levels and not just related to the kidney disease. Further research should therefore measure physical activity in control group in order to make comparison between CKD and healthy people and ideally, although much more difficult, in a CKD with different levels of physical activity. The endurance performance was improved despite no changes in VO_2max after resistance training (Mikkola et al. 2011). A similar possible effect here cannot be confirmed since endurance performance was not measured in this study. Further research should attempt a broader suite of measurements of performance in patients with CKD.

The activity of NKA skeletal muscle was not measured in this thesis due to methodological concerns (seen section 2.2.5). Given the role of phospholemman (PLM) phosphorylation in increasing the NKA activity, further research with additional measurements of both PLM and measures of NKA activity following immobilisation and exercise training would provide important insights into the NKA function in skeletal muscle.

The mechanisms which cause decreased or increased NKA content in skeletal muscle in response to immobilisation, testosterone suppression and exercise training are not fully understood. One possible mechanism is via the non-ion transducing role of the NKA α_1 isoform (Aperia et al. 2016; Xie and Askari 2002; Xie and Cai 2003). Thus further research on adaptability of NKA in skeletal muscle should investigative a possibility link with the non-ion transducing role of NKA α_1 isoform to determine the potential signalling pathways involved, and also whether these are of importance in human skeletal muscle.

An important consideration with the analysis of muscle NKA content in studies 1, 3 and 4 is the potential associated effects of either reduced (immobilisation) or increased (resistance training, recovery) on the overall muscle cross sectional area and individual

fibre size. In porcine muscle, a greater muscle sarcolemmal membrane area relative to intracellular area was associated with a higher NKA content (Harrison et al., 1994). Hence, differences in muscle fibre size could potentially also affect measurements of NKA content in human skeletal muscle. Future studies should therefore investigate the association between muscle membrane area, muscle fibre size and muscle NKA content in skeletal muscle. Given the important role of $[K^+]$ in muscle fatigue, future research should therefore also focus on K^+ regulation in the muscle cell interstitium and in plasma and the potential implications for fatigue during exercise and after resistance training.

A decrease in testosterone has been shown to increase total fat mass (Kvorning et al. 2006). Given that the [^{3}H]ouabain binding site content is expressed per gram of muscle without taking into account the amount of fat mass as part of the tissue, it is quite possible that this might also influence the measured NKA content. If so, the effect would be to arte-factually lower the detected [^{3}H]ouabain binding site content in muscle. Further research is therefore required to determine the effects of reduced testosterone on the amount of fat mass in the muscle sample and expression of the measured [^{3}H]ouabain binding site content per gram of protein (fat free mass).

Finally, in this thesis the NKA isoform abundances were measured using whole muscle homogenates which is of mixed fibre type composition. Future research should in addition focus on the possible fibre-specific adaptability of NKA isoforms in muscle single fibres.

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Appendices

Appendix 1 Individual data for Study 1 (Chapter 3) Effects of hind limb immobilisation and castration on [^{3}H]ouabain binding site content and NKA isoform abundance in rat soleus muscle

Appendix 1.1 [^{3}H]ouabain binding site content (pmol.g wet weight^{-1})

Sham group

	Control	**Non-cast leg +0**	**Cast leg +0**	**Non- cast leg +14**	**Cast leg +14 d**
1	303	476	334		
2	411	313	226		375
3	616	385	288	447	527
4	369	365	224	489	583
5	424	355	448	322	445
6	502	534	292	416	455
7	572	485		427	445
8	477	357	320	355	320
n	8	8	8	6	7
average	459	402	305	409	450
stdev	104	79	76	61	88
SEM	37	28	29	25	33

castration group

	Control	**Non-cast leg +0**	**Cast leg +0**	**Non- cast leg +14**	**Cast leg +14 d**
1	467	355	271	240	209
2	396	276	382	245	395
3	382	357	276	443	360
4	484	327	275	388	175
5	485	446	337	359	386
6	419	370	239	219	270
7	535	358		358	227
8		282	290	359	382
n	7	8	7	8	7
average	453	346	296	326	301
stdev	55	54	48	81	90
SEM	21	19	18	29	32

Appendix 1.2 Muscle NKA α_1 isoform abundance (arbitrary units a.u.)

Sham group

	Control	Non-cast leg +0	Cast leg +0	Non-cast leg +14	Cast leg +14 d
1	1.05	1.22	1.66		
2	1.09	1.48	1.72	1.93	0.91
3	1.16	1.22	0.92	0.78	1.23
4	1.21	1.35	1.33	0.86	1.25
5		1.11	0.85	1.29	0.89
6	1.01	1.51	0.53	0.94	0.84
7	0.88	0.81	0.72	1.55	0.91
8	1.26	1.14	1.21	0.84	1.47
n	7	8	8	7	7
average	1.10	1.23	1.12	1.17	1.07
stdev	0.13	0.23	0.44	0.44	0.24
SEM	0.05	0.08	0.15	0.16	0.09

Castration group

	Control	Non-cast leg +0	Cast leg +0	Non-cast leg +14	Cast leg +14
1	0.98	1.18	1.20		
2	1.13	1.08	1.72	0.98	1.12
3	1.18	1.20	1.71	0.90	1.07
4	0.91	0.93	0.49	1.37	1.15
5	0.74	1.16	0.80	0.74	1.20
6	1.10			0.84	1.35
7	0.96	1.54	0.72	0.91	1.15
8		0.89	0.50	1.12	0.86
n	7	7	7	7	7
average	1.00	1.14	1.02	0.98	1.13
stdev	0.15	0.21	0.53	0.21	0.15
SEM	0.06	0.08	0.20	0.08	0.05

Appendix 1.3 Muscle NKA α_2 isoform abundance (arbitrary units a.u.)

Sham group

	Control	Non-cast leg +0	Cast leg +0	Non-cast leg +14	Cast leg +14 d
1	1.35	1.32	2.32	2.64	
2	1.85	3.03	2.58	2.23	1.92
3	2.87	0.32	1.21	1.23	1.51
4	0.64	0.47	0.37	0.49	1.51
5	1.53	1.91	0.95	1.59	0.50
6	1.40	1.96	0.12	0.99	0.44
7	1.01	0.89	0.60	1.15	0.60
8	1.45	1.24	1.09	0.75	1.42
n	8	8	8	8	7
average	1.51	1.39	1.16	1.38	1.13
stdev	0.66	0.89	0.88	0.73	0.60
SEM	0.23	0.32	0.31	0.26	0.23

Castration group

	Control	Non-cast leg +0	Cast leg +0	Non-cast leg +14	Cast leg +14
1	1.57	2.25	1.21		0.74
2	2.76	1.02			
3	1.52	1.37	0.68		0.66
4	0.64	1.71	0.36	0.71	0.96
5	1.68	1.75	0.49	0.74	0.90
6	0.44	1.04	0.38	0.58	1.08
7	0.97	0.51	0.30	0.67	0.94
8			0.40	0.93	0.29
n	7	7	7	5	7
average	1.37	1.38	0.55	0.73	0.80
stdev	0.78	0.58	0.32	0.13	0.26
SEM	0.30	0.22	0.12	0.06	0.10

Appendix 1.4 Muscle NKA α_3 isoform abundance (arbitrary units a.u.)

Sham group

	Control	Non-cast leg +0	Cast leg +0	Non-cast leg +14	Cast leg +14 d
1	1.40	0.72	1.17	1.95	
2	0.62	1.24	1.38	0.00	1.14
3	0.71	0.46	1.33	1.32	1.36
4	0.95	1.29	0.19	0.54	1.36
5	1.98	1.16	0.93	1.71	0.93
6	0.94	1.10	0.35	0.58	0.39
7	1.58	1.14	1.91	1.10	0.96
8	1.02	1.54	0.95	0.82	1.25
n	8	8	5	6	6
average	1.15	1.08	1.03	1.00	1.06
stdev	0.46	0.34	0.56	0.65	0.34
SEM	0.16	0.12	0.20	0.23	0.13

Sham group

	Control	Non-cast leg +0	Cast leg +0	Non-cast leg +14	Cast leg +14
1	1.03	0.66		0.32	0.32
2	1.24	1.38			
3	0.36	1.29		0.77	0.62
4	0.19	0.59	0.30	1.01	1.01
5	0.95	1.42	0.81	0.86	0.86
6	1.96	0.63	0.50	0.57	0.57
7	1.53	0.79	1.07	1.04	1.04
8	0.57	0.73	1.40	0.56	0.56
n	8	8	5	7	7
average	0.98	0.93	0.81	0.73	0.71
stdev	0.60	0.36	0.44	0.26	0.27
SEM	0.21	0.13	0.20	0.10	0.10

Appendix 1.5 Muscle NKA β_1 isoform abundance (arbitrary units a.u.)

Sham group

	Control	Non-cast leg +0	Cast leg +0	Non-cast leg +14	Cast leg +14 d
1	0.84	0.77	0.73	0.98	
2	0.70	1.06	1.09	0.97	0.85
3	1.66	0.63	1.48	1.04	1.20
4	0.74	0.77	0.58	0.91	1.20
5	0.50	1.10	0.94	0.94	0.14
6	0.73	0.98	0.14	0.88	0.30
7	0.49	0.54	0.88	1.13	0.40
8	0.69	0.86	0.74	0.85	0.90
n	8	8	8	8	7
average	0.79	0.84	0.82	0.96	0.71
stdev	0.37	0.20	0.39	0.09	0.43
SEM	0.41	0.22	0.43	0.10	0.39

Castration group

	Control	Non-cast leg +0	Cast leg +0	Non-cast leg +14	Cast leg +14
1	0.45	0.93	0.58		0.42
2	0.84	1.06	1.09		
3	1.48	1.04	0.82		0.87
4	0.74	0.58	0.56	1.00	0.95
5	1.11	1.31	0.46	0.64	0.52
6	0.23	0.98	0.33	0.62	0.60
7	0.49	0.92	0.59	0.57	0.46
8		0.41	0.45	0.76	0.24
n	7	8	8	5	7
average	0.76	0.90	0.61	0.72	0.58
stdev	0.43	0.29	0.24	0.17	0.25
SEM	0.16	0.10	0.09	0.08	0.10

Appendix 1.6 Muscle NKA β_2 isoform abundance (arbitrary units a.u.)

Sham group

	Control	Non-cast leg +0	Cast leg +0	Non-cast leg 14	Cast leg +14 d
1	1.02	0.34	0.62	1.97	
2	0.74	1.64	0.88	0.84	1.53
3	1.74	0.14	1.58	0.63	1.51
4	0.24	0.10	0.21	0.53	1.51
5	0.50	1.10	0.94	0.94	0.14
6	0.73	0.98	0.14	0.88	0.30
7	1.77	0.95	0.13	1.31	0.68
8	0.83	0.66	1.00	0.56	1.65
n	8	8	8	8	7
average	0.95	0.74	0.69	0.96	1.05
stdev	0.55	0.53	0.51	0.48	0.65
SEM	0.19	0.19	0.18	0.17	0.25

Castration group

	Control	Non-cast leg +0	Cast leg +0	Non-cast leg +14	Cast leg +14
1	0.89	1.63	0.17		0.17
2	0.83	1.64	0.88		
3	1.66	1.12	0.23		0.32
4	0.24	0.21	0.21	0.83	0.92
5	1.07	1.54	0.18	1.03	.50
6	1.13	0.43	0.18	0.70	0.88
7	0.48	1.01	0.20	0.50	0.24
8		0.23	0.20	0.65	0.39
n	8	8	7	5	6
average	0.90	0.98	0.28	0.74	0.63
stdev	0.46	0.62	0.24	0.20	0.49
SEM	0.17	0.22	0.09	0.09	0.18

Appendix 2 Individual data for study 2 (Chapter 4) Effects of sprint training on skeletal muscle NKA content and isoform abundance in humans

Appendix 2.1 [^{3}H]ouabain binding site content (pmol.g wet weight^{-1})

Control

Subject	Pre	Post
1	479	426
2	330	313
3	194	204
4	331	345
5	402	324
6	360	460
7	283	301
n	7	7
average	340	339
stdev	89	84
SEM	34	32

Sprint training

Subject	Pre	Post
1	412	336
2	345	426
3	315	388
4	415	497
5	351	385
6	158	452
7	301	621
8	241	157
n	8	8
average	317	408
stdev	86	133
SEM	31	47

Appendix 2.2 Muscle NKA α_1 and α_2 isoform abundances (arbitrary units a.u.)

α_1 isoform

	Control		Sprint training	
Subject	**Pre**	**Post**	**Pre**	**Post**
1	1.21	1.20	0.71	2.03
2	1.64	0.98	0.40	0.58
3	1.14	0.48	1.65	0.66
4	0.28	0.91	0.92	3.07
5	0.32	0.76	0.87	0.67
6	0.42	0.77	0.66	0.83
7	0.35	0.38	1.16	0.45
8			0.82	0.81
n	7	7	8	8
average	0.76	0.78	0.90	1.14
stdev	0.55	0.28	0.37	0.92
SEM	0.21	0.11	0.13	0.33

α_2 isoform

	Control		Sprint training	
Subject	**Pre**	**Post**	**Pre**	**Post**
1	1.09	0.82	0.79	2.08
2	2.11	1.14	0.68	0.64
3	1.31	0.57	1.58	0.67
4	0.31	0.55	1.07	2.69
5	0.52	0.48	0.87	0.67
6	0.47	0.66	1.12	1.01
7	0.60	0.54	0.69	0.37
8			1.14	0.85
n	7	7	8	8
average	0.92	0.68	0.99	1.12
stdev	0.64	0.23	0.30	0.82
SEM	0.24	0.09	0.11	0.29

Appendix 2.3 Muscle NKA β_1 and β_2 isoform abundances (arbitrary units a.u.)

β_1 isoform

	Control		Sprint training	
Subject	**Pre**	**Post**	**Pre**	**Post**
1	1.45	0.95	0.85	1.57
2	0.72	0.58	0.77	0.89
3	1.02	0.74	1.00	0.65
4	0.36	0.97	0.97	2.05
5	0.73	0.40	0.70	0.60
6	0.55	0.36	1.60	1.78
7	0.41	0.48	1.34	0.51
8			1.08	0.93
n	7	7	8	8
average	0.75	0.64	1.04	1.12
stdev	0.38	0.25	0.30	0.59
SEM	0.14	0.09	0.11	0.21

β_2 isoform

Subject	**Pre**	**Post**	**Pre**	**Post**
1	1.44	1.46	0.46	1.56
2	1.86	1.33	0.73	0.82
3	1.33	0.66	1.62	0.91
4	0.56	0.85	0.80	1.77
5	0.39	0.28	1.36	0.88
6	0.48	0.23	1.38	1.55
7	0.24	0.39	1.14	0.36
8			1.59	0.80
n	7	7	8	8
average	0.90	0.74	1.13	1.08
stdev	0.63	0.50	0.43	0.49
SEM	0.24	0.19	0.15	0.17

Appendix 3 Individual data for study 3 (chapter 5) Effects of resistance training on skeletal muscle NKA content and isoform abundance

Appendix 3.1 [^{3}H]ouabain binding site content (pmol.g wet weight^{-1})

	Control		**Resistance training**	
subject	**Pre**	**Post**	**Pre**	**Post**
1	417	412	412	427
2	222	296	259	302.
3	377	345	411	307
4	331	363	317	272
5	235	227	287	396
6			189	235
7			350	393
8			320	442
9			304	342
10			374	377
11			308	314
12			441	520
13			221	268
14			238	312
15			##	##
16			##	##
n	5	5	16	16
average	316	329	311	349
stdev	86	70	76	76.
SEM	38	31	20	20

Appendix 3.2 Muscle NKA α_1 isoform abundance (arbitrary units a.u.)

Control			**Resistance training**	
subject	**Pre**	**Post**	**Pre**	**Post**
1	1.21	1.37	0.84	0.92
2	1.07	1.11	0.79	1.38
3	0.93	1.18	1.07	2.43
4	0.99	1.26	1.42	1.03
5	0.77	0.48	1.68	1.47
6			1.81	1.41
7			1.14	2.11
8			0.76	1.60
9			1.44	2.63
10			1.46	1.69
11			1.07	1.62
12			0.99	2.62
13			1.30	1.16
14			0.95	1.11
15			1.21	1.35
16			1.20	1.00
n	5	5	16	16
average	0.99	1.08	1.20	1.59
stdev	0.17	0.35	0.31	0.57
SEM	0.07	0.16	0.08	0.14

Appendix 3.3 Muscle NKA α_2 isoform abundance (arbitrary units a.u.)

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	Control		Resistance training	
subject	**Pre**	**Post**	**Pre**	**Post**
1	1.86	1.40	1.74	1.91
2	1.88	1.94	1.86	2.05
3	0.82	0.96	1.97	2.17
4	0.63	0.71	1.16	1.28
5	0.89	0.82	1.40	1.54
6			1.86	2.05
7			1.43	1.58
8			0.98	1.08
9			1.81	1.99
10			1.59	1.75
11			1.93	2.12
12			1.53	1.69
13			1.05	1.16
14			1.01	1.11
15			1.23	1.36
16			0.91	1.00
n	5	5	16	16
average	1.22	1.17	1.47	1.61
stdev	0.60	0.51	0.36	0.41
SEM	0.27	0.23	0.09	0.10

Appendix 3.4 Muscle NKA β_1 isoform abundance (arbitrary units a.u.)

	Control		Resistance training	
subject	**Pre**	**Post**	**Pre**	**Post**
1	0.71	0.51	1.99	1.70
2	1.97	2.47	2.15	1.83
3	0.59	0.39	2.34	2.32
4	1.63	2.00	1.55	1.30
5	0.55	0.49	1.01	0.98
6			1.29	1.68
7			1.26	1.01
8			1.43	1.71
9			0.90	1.37
10			1.18	0.83
11			1.61	2.01
12			1.65	1.31
13			1.38	1.38
14			1.28	1.06
15			1.49	0.63
16			1.04	1.14
n	5	5	16	16
average	1.09	1.17	1.47	1.39
stdev	0.66	0.99	0.41	0.46
SEM	0.29	0.44	0.10	0.11

Appendix 3.5 Muscle NKA β_2 isoform abundance (arbitrary units a.u.)

	Control		Resistance training	
subject	**Pre**	**Post**	**Pre**	**Post**
1	1.73	2.20	1.45	1.22
2	2.32	1.71	1.13	0.95
3	1.00	0.47	1.67	1.74
4	0.53	0.41	1.29	1.21
5	0.34	0.56	1.43	1.25
6			1.90	1.07
7			1.09	0.96
8			1.36	1.36
9			1.37	1.36
10			1.71	1.25
11			1.33	1.33
12			1.47	1.47
13			1.15	1.34
14			1.07	0.95
15			1.29	1.83
16			1.22	1.16
n	5	5	16	16
average	1.18	1.07	1.42	1.28
stdev	0.83	0.83	0.23	0.25
SEM	0.37	0.37	0.06	0.09

Appendix 4 Individual data for study 3 (chapter 5) Effects of moderate and high intensity training on skeletal muscle NKA content and isoform abundances in patients with chronic kidney disease

Appendix 4.1 [^{3}H]ouabain binding site content relative abundance (pmol.g wet weight^{-1})

subject	CKD	Healthy CON
1	337	294
2	291	351
3	282	362
4	359	291
5	311	352
6	377	343
7	287	328
8	289	319
9	329	299
10	230	321
11	306	315
12	334	290
13	288	308
14	294	329
15	302	292
n	15	15
average	312	320
stdev	38	24
SEM	10	76

Appendix 4.2 Muscle NKA α_1 and α_2 isoform abundances (arbitrary units a.u.)

	α_1 isoform		α_2 isoform	
subject	**CKD**	**Healthy CON**	**CKD**	**Healthy CON**
1	0.65	0.76	0.66	0.96
2	0.93	0.82	0.83	0.78
3	0.62	0.70	0.84	0.70
4	0.77	0.94	0.90	0.87
5	1.14	0.67	0.85	1.44
6	0.50	0.86	0.86	0.92
7	1.23	0.61	0.79	0.83
8	0.30	0.58	0.90	0.88
9	0.88	1.01	0.92	0.85
10	0.65	0.73	0.86	0.94
11	1.08	0.79	1.02	0.91
12	0.99	1.09	0.90	1.01
13	0.68	0.65	0.71	0.88
14	0.75	0.88	1.11	0.95
15	0.84	0.90	0.63	0.81
n	15	15	15	15
average	0.80	0.80	0.85	0.92
stdev	0.28	0.15	0.07	0.17
SEM	0.07	0.04	0.02	0.04

Appendix 4.3 Muscle NKA β_1 and β_2 isoform abundances (arbitrary units a.u.)

β_1 isoform			β_2 isoform	
subject	**CKD**	**Healthy CON**	**CKD**	**Healthy CON**
1	0.96	0.86	0.79	1.04
2	0.85	0.93	1.11	0.93
3	0.75	0.80	0.76	1.03
4	0.71	0.87	0.82	1.02
5	0.95	0.94	1.01	0.94
6	0.84	0.90	1.07	0.92
7	1.17	0.85	1.21	1.00
8	0.72	0.81	0.60	0.92
9	0.64	0.82	0.86	0.95
10	0.95	0.81	0.94	0.71
11	0.97	0.86	1.35	0.90
12	1.11	0.95	1.27	0.92
13	1.18	0.91	1.24	0.98
14	1.16	0.85	1.02	0.85
15	1.01	0.97	1.02	0.97
n	15	15	15	15
average	0.93	0.88	1.00	0.94
stdev	0.15	0.06	0.17	0.08
SEM	0.04	0.01	0.05	0.03

Appendix 4.4 [^{3}H]ouabain binding site content (pmol.g wet weight^{-1})

	CKD-CON		MICT		HIIT	
subject	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
1	337	303	282	307	289	295.
2	291	309	359	312	329	313.
3			311	317	230	282.
4			377	352	306	322
5			287	256	334	307
6					288	305
7					294	300
8					302	290
n	2	2	5	5	8	8
average	314	306	323	309	305	302
stdev	32	5	43	34	17	13
SEM	22	3	19	15	6	5

Appendix 4.5 Muscle NKA α_1 and α_2 isoform abundances (arbitrary units a.u.)

α_1 isoform

	CKD-CON		MIT		HIT	
subject	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
1	0.65	0.78	0.62	0.69	0.30	0.93
2	0.93	0.87	0.77	0.99	0.88	1.23
3			1.14	0.65	0.65	0.21
4			0.50	0.99	1.08	0.65
5			1.23	1.11	0.99	0.61
6					0.68	1.22
7					0.75	0.68
8					0.84	1.02
n	2	2	5	5	8	8
average	0.79	0.82	0.85	0.89	0.77	0.82
stdev	0.20	0.06	0.32	0.20	0.24	0.35
SEM	0.14	0.04	0.14	0.09	0.08	0.12

α_2 isoform

	CKD-CON		MIT		HIT	
subject	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
1	0.66	0.90	0.84	0.83	0.90	0.87
2	0.83	0.64	0.90	0.90	0.92	1.13
3			0.85	0.98	0.86	0.83
4			0.86	0.89	1.02	0.96
5			0.79	1.06	0.90	0.85
6					0.71	0.84
7					1.11	1.09
8					0.63	0.91
n	2	2	5	5	8	8
average	0.75	0.77	0.85	0.93	0.88	0.94
stdev	0.11	0.18	0.04	0.09	0.15	0.12
SEM	0.08	0.13	0.02	0.04	0.05	0.04

Appendix 4.6 Muscle NKA β_1 and β_2 isoform abundances (arbitrary units a.u.)

β_1 isoform

subject	CON		MIT		HIT	
1	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
2	0.96	0.83	0.75	0.64	1.01	1.17
3	0.85	0.78	0.71	0.74	0.64	0.92
4			0.95	0.86	1.18	1.09
5			0.84	1.03	0.97	0.93
6			1.17	1.02	1.11	1.17
7					0.95	0.99
8					1.16	1.15
					0.72	0.61
n	2	2	5	5	8	8
average	0.90	0.81	0.88	0.86	0.96	0.98
stdev	0.08	0.04	0.18	0.17	0.21	0.19
SEM	0.05	0.03	0.08	0.08	0.08	0.07
	0.750					

β_2 isoform

	CON		MIT		HIT	
subject	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
1	0.79	0.73	0.76	0.64	0.76	0.64
2	1.11	0.96	0.82	0.88	0.82	0.88
3			1.01	0.98	1.01	0.98
4			1.07	0.99	1.07	0.99
5			1.21	1.02	1.21	1.02
6					1.24	1.07
7					1.02	1.11
8					1.02	1.16
n	2	2	5	5	8	8
average	0.95	0.85	0.97	0.90	1.02	0.98
stdev	0.22	0.16	0.18	0.16	0.17	0.16
SEM	0.16	0.12	0.08	0.07	0.06	0.06

Appendix 5 Resistance training One-repetition maximum (1RM) method and results

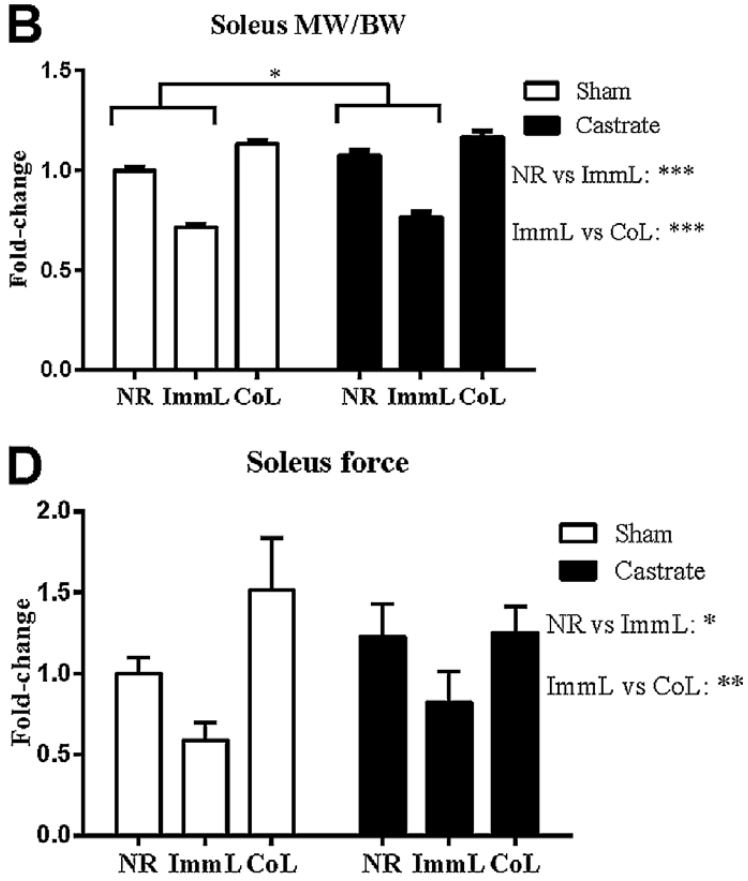
One repetition maximum test (1RM)

Baseline the weight load for the training comprised of two maximal strength tests (bench press and leg press exercises). Each participant performed three sets, at 50%, 70%, 90% effort, each with two tosix repetitions, followed by one set to failure of one to four repetitions, for each of the bench press and leg press exercises. Each set to failure was used to predict the one repetition maximum (1RM.

Results

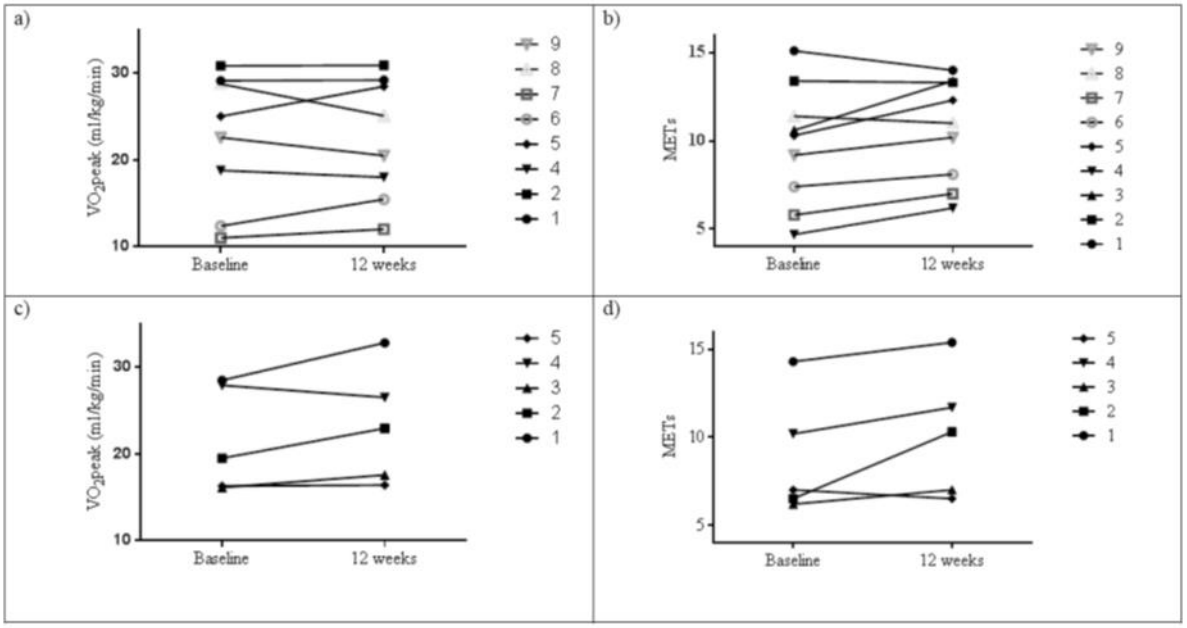
	Leg Press								Bench Press						
CON	**3**	**6**	**9**	**12**	**15**	**18**	**21**		**3**	**6**	**9**	**12**	**15**	**18**	**21**
1	243.3	286.9	308.7	352.4		327.1	374.2		70.9	74.6	69.4	77.1		69.4	74.6
2	278.5	260.2	310.2	286.9	331.4	332.2	327.1		64.3	72	70	74.6	77.1	79.4	79.7
3	402.8	424.1	507.7	492.8	459	511.3	537.7		84.5	87.3	90	92.7	87.4	95.3	95.1
4	330.6	341.5	385.1	428.8	461.5	456	494.2		79.4	84.6	84.7	80	80	84.9	82.3
5	318.4	392.6	459.7	430	501.7	461.5	531.7		90	84.7	80	84.9	92.6	87.4	87.5
6	495.6		532.2	511.4	532.6	553.8	657.9		115		115	121.8	118.3	127.1	132.4
7	329.7	341.5	330.6	352.4	358		363.1		65	65	63.5	67.5	63.5		66.9
8	308.7	341.5	368.8	391.5	408.4		429.7		66.9	69.4	69.4	76.8	81.8		72.5
	338.5	**341.2**	**400.4**	**405.8**	**436.1**	**440.3**	**464.5**		**79.5**	**76.8**	**80.3**	**84.4**	**85.8**	**90.6**	**86.4**
	78.3	**56.3**	**88.7**	**75.5**	**73.7**	**92.9**	**111.3**		**17.2**	**8.7**	**16.7**	**16.8**	**17.0**	**19.9**	**20.6**
CWI															
1	363.3	385.9		363.3	379.7	396	405.5		50	55		54.4	61.7	61.7	66.9
2	368.8	412.4			450.6	488.8	546.5		80	77.1			79.7	82.3	85
3	341.5		352.4	363.3	352.4	363.2	383.7		70.9		68.8	68.8	71.5	74.1	70
4	297.8	319.7		363.3	348		397.2		57.5	56.6		58.2	60.7		65.5
5	289.1		363.3		406.9	428.8	550.4		74.1		81.8			84.9	92.7
6	352.4	368.2	378	439.7	445.4	494.2	490.3		97.7	97.9	102.9	108	102.9	105.9	105
7	297.8		363.4		363.2	374.2	385.1		87.3		84.7		84.7	82.3	87.3
8	459	485.5	531.7	488.8	579.7	585.7	684.2		87.4	87.5	90	92.7	98.2	92.5	95
	346.2	**394.3**	**397.8**	**403.7**	**415.7**	**447.3**	**480.4**		**75.6**	**74.8**	**85.6**	**76.4**	**79.9**	**83.4**	**83.4**
	55.5	**61.2**	**75.4**	**58.0**	**77.2**	**80.1**	**108.1**		**16.0**	**18.9**	**12.4**	**23.1**	**16.6**	**13.9**	**14.5**

Appendix 6 Impact of immobilization and castration on rat soleus mass and strength



Impact of immobilization and castration on soleus mass and strength. Soleus MW/BW (B), soleus muscle force (D) were examined for muscles isolated from NR/ImmL and the CoL of immobilized rats with sham/castration surgery. *p≤ 0.05,**p≤ 0.01, and **p≤0.001 in two-way ANOVA analysis. Symbols of p value of Castration main effect are illustrated on top of columns; symbols of p value of Immobilization main effects ("NR vs ImmL" and "ImmL vs CoL") are illustrated on the right side of each panel. MW/BW = muscle weight/body weight; NR = non-immobilized rats; Imm = immobilized rats; CoL = ontralateral leg. From (Xuzhu Lin et . al 2016),

Appendix 7 CKD study peak oxygen uptake (VO_2peak) results



Appendix 8 Sprint training study/ sprint performance methods and results

Methods

Wingate test

The Wingate test consisted of a 30-s maximal sprint against constant resistance. For each subject the load was determined according to body mass using the optimisation tables of Bar-Or (0.087 kg × kg-1 body mass). Strong motivation was given verbally to subjects during the test. Seat height was adjusted to each subject's satisfaction and toe-clips were used to prevent the subject's feet from slipping off the pedals. This height was recorded and kept the same for each subject in all trials. Peak power is the highest mechanical power elicited during the test. This index was taken as the highest average power during any 3 - 5-s period. Mean power is the average power sustained throughout the 30-s period. For both the F-V and Wingate tests, the characteristics of the ergocycle allowed the calculation of Pmax, Ppeakand Pmean as follows: 1) for the F-V test: P (W) = Fopt (kg) × Vopt (rev × min-1) and 2) for the Wingate test: P (W) = F (kg) × V (rev × min-1).

Results

Post training

Subject	Duration (sec)	Torque (Nm)	Torque factor (Nm/Kg)	Peak Power (W)	Mean Power (W)	Minimum Power (W)
1	30.01	74.20	0.70	1012.21	747.78	301.22
2	30.03	66.22	0.77	1217.14	702.05	566.46
3	30.01	61.60	0.77	1159.43	574.58	292.50
4	30.01	53.20	0.70	711.45	571.36	419.41
5	30.00	67.20	0.70	1543.43	601.55	330.77
6	30.00	48.91	0.67	905.53	442.42	325.27
7	30.00	49.70	0.70	869.57	696.87	552.60
8	30.00	41.07	0.74	457.03	291.36	218.87
n	8	8	8	8	8	8
mean	30.01	57.76	0.72	984.48	578.50	375.89
SD	0.01	11.26	0.04	331.69	150.95	126.05

Pre training

Subject	Duration (sec)	Torque (Nm)	Torque factor (Nm/Kg)	Peak Power (W)	Mean Power (W)	Minimum Power (W)
1	30.00	74.20	0.70	972.81	714.44	268.59
2	30.01	66.22	0.77	825.78	702.77	485.40
3	30.01	60.83	0.77	1399.73	701.94	274.35
4	30.01	58.90	0.77	911.42	573.62	288.75
5	30.00	72.80	0.70	1094.30	658.32	676.68
6	30.01	54.76	0.74	555.28	443.71	369.50
7	30.01	49.70	0.70	1322.51	597.48	367.35
8	30.00	41.07	0.74	597.10	330.72	230.73
n	8	8	8	8	8	8
mean	30.01	59.81	0.74	959.87	590.37	370.17
SD	0.01	11.32	0.03	306.79	138.54	147.75