A ROLE FOR NCAM IN THE AGE OLD STORY OF SARCOPENIA

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ABSTRACT

Sarcopenia is a major contributor to the loss of independence and deteriorating quality of life in elderly individuals. It is marked by declining skeletal muscle mass and strength beyond the age of 65. Sarcopenia places significant financial and social burdens on societies, particularly those with ageing populations such as New Zealand. Therefore, research into the pathogenesis of this condition is essential if we are to delay its onset and reduce its significance to our society.

The current hypotheses suggest age-associated loss of muscle follows the permanent loss of motoneuron synaptic contact (denervation). The failure of neighbouring intact neurons to compensate (re-innervation) results in death or atrophy of the fibre. I propose that the loss of re-innervative ability is the result of age-related down regulation of a neurite attracting factor (NAF) released from muscle fibres upon denervation. Neural cell adhesion molecule (NCAM) is a prime candidate protein for this proposed NAF as it is an essential mediator of nerve growth and synaptic contact during development. More recently NCAM cell adhesion and intracellular signalling roles have both been identified based on the post-translational addition of polysialic acid (PSA) to NCAM. Therefore, the following investigation aimed to first replicate previous work showing that young muscle fibres produce high levels of NCAM in response to denervation. Second, I aimed to determine if an age-related decline in NCAM up-regulation exists in response to denervation in elderly muscle. Young animals responded to denervation with significantly increased NCAM present at extra-junctional locations in the muscle fibre. There were significant extra-junctional increases in un-polysialylated NCAM (unNCAM) (14.1 - 20.5 relative protein level), while polysialylated NCAM (PSA-NCAM) showed significant cytoplasmic increases (19.9 - 31.7 relative protein level). By contrast, elderly animals showed 8.7% less extra-junctional unNCAM and 22.5% less cytoplasmic PSA-NCAM than young animals.

These observations suggest that an age-related decline in the production of NCAM in response to denervation may be a key regulator of the inability of muscle fibres to attract a neuronal sprout. Therefore, I believe that reduced ability to respond to denervation by up-regulation of NCAM production may be a primary contributor to the problem of long-term denervation among elderly muscle fibres. Further investigation
needs to focus on what specific NCAM regulatory signalling pathways are impaired in elderly muscles so that in future we may develop a strategy to keep or restore neuronal input to elderly muscle fibres, thereby preventing much of the age-related muscle atrophy.
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**TERMS & ABBREVIATIONS**

**C**

- C57/B6j
  - Mouse strain used in current investigation
- Ca^{2+}
  - Calcium
- CH$_3$COSCH$_2$CH$_2$N(CH$_3$)$_3$I
  - Acetylthiocholine iodide
- CNS
  - Central nervous system
- CuSO$_4$.5H$_2$O
  - Copper sulfate

**D**

- DEDL
  - Denervated extensor digitorium longus
- Dok7
  - Docking protein 7

**E**

- ECM
  - Extracellular matrix
- EDL
  - Extensor digitorium longus
- EDL I
  - Innervated extensor digitorium longus

**F**

- FNIII
  - Fibronectin 3

**G**

- GFAP
  - Glial fibrillary acidic protein
- GPI
  - Glycophosphatidylinositol
H

H₂O
Distilled water
HIER
Heat induced epitope retrieval
HO₂CCHCHCO₂H
Maleic acid

I

Ig
Immunoglobulin
IHC
Immunohistochemistry
IU
international unit

K

K₃[Fe(CN)₆]
Potassium ferricyanide
KCl
Potassium chloride
kDa
Kilodaltons
KH₂PO₄
Potassium hydrogen phosphate
KO
Knockout

L

L
Litre
LED
Light emitting diode
Lrp4
Low density lipoprotein receptor-related protein 4

M

mAChR
Muscarinic acetylcholine receptors
mM
Milligrams
MHC
Myosin heavy chain
mL
Millilitres
mmol.L⁻¹
Millimoles per litre
MRI
Magnetic resonance imaging
mRNA
Messenger ribonucleic acid
ms⁻¹
Metres per second
Musk
Muscle-specific receptor tyrosine kinase
NT4
  Neurotrophin 4

O
  O.C.T
    Optimal cutting temperature
  OVN
    Overnight

P
  PBS
    Phosphate buffered saline
  PC
    Post conception
  PKC
    Protein kinase C
  PLCγ
    Phospholipase C gamma
  PNS
    Peripheral nervous system
  PPP
    Protein per pixel
  PSA
    Polysialic acid
  PSA-NCAM
    Polysialylated neural cell adhesion molecule
  PST
    Polysialictransferase ST8Sia IV

R
  RyR1
    Ryanodine receptor

S
  SBH
    Sodium borohydrate
  SDS
    Sodium dodecyl sulfate
  SeeDB
    See deep brain
  SolD
    Denervated soleus
  SolI
    Innervated soleus
  SD
    Standard deviation
  STX
    polysialictransferase ST8Sia II
  SYN
    Synaptophysin

T
  TA
    Tibialis anterior
  TBS
    Tris buffered saline

U
  unNCAM
    Unpolysialylated neural cell adhesion molecule

W
  WGA
    Wheat germ agglutinin
1. CHAPTER ONE

INTRODUCTION
1.1 Preface

1.1.1 What is sarcopenia?

In youth, we dream about the benefits and independence of age; in age, we reminisce about the strength and self-sufficiency of youth. Ageing is an inevitable process that can result in loss of the independence we once experienced, due to sarcopenia. Sarcopenia is defined as the age-related loss of skeletal muscle mass due both to atrophy of muscle fibres (reduction in the size of the fibres) (Figure 1.01A) and/or reduction in the number of muscle fibres (Figure 1.01B). Sarcopenia is a multifaceted condition associated with other pathophysiological manifestations including loss of bone mass (osteoporosis) (Wattjes & Fischer, 2013), loss of muscle strength (dyapenia) (Kwan, 2013b) and obesity (Wattjes & Fischer, 2013) (Baumgartner et al., 2004). The clinical onset of sarcopenia remains undefined as the effects of muscle loss differ from subject to subject, however previous investigations suggest lean muscle mass declines by approximately 1% per year from age 40 (Janssen et al., 2000), culminating in significant negative effects on day to day functioning and independence beyond the age of 65.

Skeletal muscle represents approximately 40% of the entire human tissue mass, with muscles being formed from collections of long multinucleated fibres (Ohlendieck, 2010). The reduction in myofibre size (atrophy) and number that is apparent from age 40 may be masked by the age associated infiltration of intramuscular adipose tissue (Lexell et al., 1988) resulting in a reduction in muscle mass often without an obvious reduction in Body Mass Index (BMI) (Figure 1.01a). This loss of muscle mass and strength...
contributes to an increasingly sedentary lifestyle among ageing individuals, exacerbating the weakness associated with muscle loss, contributing to a spiralling decline in independence and potential reduction in quality of life.

**1.1.2 Social and economic impact of sarcopenia**

The significance of societal effects of declining independence with age become apparent when considering the 2014 New Zealand (NZ) census data (Figure 1.02). The demographic of NZ is changing population features a doubling of the 65 years plus cohort in the next 40 years (by 2051) (Bascand, 2012; MacPherson, 2014), (Figure 1.02A/B.). This shift arises partly by a decrease in mortality rate (Fries, 2002) coupled with a steady increase of life expectancy at roughly one month annually (Johnston, 2009). NZ’s ageing population features a steadily increasing number of dependent elderly people within a community coupled with a progressively reducing proportion of taxpayers. The long-term effect of the changing demographic creates an environment of economic and potentially intergenerational discord.

The continual increase of NZ’s average life expectancy of 79 years (Organisation, 2014) coupled with reduced self-sufficiency of many people over 65 suggests more than ten years of elderly individuals lives will be spent reliant on the working population. The working population in NZ is expected to decrease due to a declining birth rate (decline of 2000 births between 2012 and 2013), suggesting that in future the working population may not be able to support the growing numbers of dependent elderly people (MacPherson, 2014) (Figure 1.02A). This changing demographic is clearly highlighted by the NZ dependency ratio of individuals 65+ years and individuals in the working population age 15 - 60 years (Figure 1.02). In 2011, NZ had five working individuals for every one aged (65+) person requiring support. However, a steady increase in this ratio projects that for every two working individuals there will be one elderly individual in need of financial support by 2061 (MacPherson, 2014). Such an elevated dependency ratio coupled with increasing life expectancy is projected to have a major effect on the economy (MacPherson, 2014).
Aged care and hospital admittance attributed to sarcopenia-associated injury are key financial contributors to the economic and social burden. The primary source of hospital admittance for patients in the 80+ age bracket in New Zealand is musculoskeletal-related conditions or fall related injuries (Cornwall & Davey, 2004). These hospital admissions alone are projected to cost $2.1 billion annually by 2021, in addition to residential aged care admissions, which are associated with an increase in dependence, costing $683 million annually (Cornwall & Davey, 2004). Together, this represents a cost of $2.8 billion annually and emphasises the importance of gaining a better understanding of the factors contributing to these outcomes.

Figure 1.02 2014 Census population data
The predicted changes in the total number and percentage of the total population that four age brackets (0 - 14, 15 - 39, 40-64 and 65+) represent within New Zealand. A) The prevalence of 65+ individuals increase from 58,000 in 2009 to 140,000 by 2061. B and C) The group aged 65+ is predicted to represent a larger percentage of the total population increasing from 12% in 2011 to 25% in 2036. D) The aged dependency ratio depicts a steady increase in the number of 65+ individuals present per 100 working individuals (15 – 64 years). The ratio is increasing from five working individuals per elderly person in 2011 to two working individuals per elderly person by 2061. Adapted from NZ statistics (MacPherson, 2014).
understanding of the physiological manifestations that underpin sarcopenia so that the direct economic and social impacts can be minimised.

1.1.3 Sarcopenic etiology

Sarcopenia affects everyone to some extent by age 65. Decreases of 10% of muscle mass are seen between age 24 - 50 increasing to 30% between age 50 - 80 (Wattjes & Fischer, 2013). This age-specific decline in skeletal muscle affects both sexes with losses of approximately 0.47% and 0.37% per annum for males and females respectively. The age-related loss of muscle mass appears to occur at a greater rate in men compared to women (Janssen et al., 2000). Despite discrepancies between gender susceptibility, both sexes show a rate of muscle mass decline that is dependent upon the rostral-caudal position of the muscle within the body. Magnetic resonance imaging (MRI) studies investigating changes in skeletal muscle mass in 18 - 88 year old male and female subjects found a larger age-related loss of skeletal muscle in the lower limbs compared to the upper limbs (Janssen et al., 2000); (Goodpaster et al., 2006). This differential decline in muscle mass and strength of the lower limbs may contribute to the increasing dependence (the state of relying on someone or something else) of the aged population.

Sarcopenia is thought to be caused by many factors including dysregulation of protein metabolism and catabolism, changes in the release of hormones (for example insulin-like growth factor-1), increases in oxidative stress following dysfunctional mitochondrial metabolism, and age-related decreases in skeletal muscle innervation (Narici & Maffulli, 2010; Mitchell et al., 2012).

Ageing coincides with muscle denervation that results from the retraction of the innervating motor axons. In young animals denervated motoneuron (α-MN) endplates are typically re-innervated via terminal or nodal (neighbouring) axonal sprouting (Cristea et al., 2010; Kwan, 2013a). Long-term denervated muscle fibres presence in old animals suggests either nerves lose their ability to regrow axons or muscle fibres lose their ability to attract or accept re-innervation. I propose that old muscles lose their ability to stimulate axon sprouts to re-attract neural input, and therefore the primary aim of this thesis is to test that hypothesis.
1.2 LITERATURE REVIEW

1.3 Skeletal muscle

1.3.1 Development

Skeletal muscle cells are one of the most abundant cell types within the mammalian body, forming muscles that represent about 40% of the entire human tissue mass (Ohlendieck, 2010). In mammals, fibres are generally subdivided into slow (containing type 1 myosin heavy chain, MHC I) and fast types (containing MHCIIa and IIx/b) (Marcell, 2003; Schiaffino et al., 2007). Development of these fibre types involves the differentiation of myogenic cells (myoblasts) into primary myotubes, secondary myotubes or satellite cells (Sheard et al., 1991). In rats this process follows a biphasic pattern involving the progressive fusion of primary and then secondary myotubes (Ross et al., 1987). The developmental process is believed to be controlled by two influences, as primary myotubes have been shown to develop autonomously, whereas myoblasts destined to form secondary myotubes require motor innervation (Ross et al., 1987). Fibre type differentiation involves stage-dependent changes in the expression of myosin heavy chain (MHC) isoforms. Embryonic primary myotubes predominantly express the embryonic MHC isoform during formation (up to embryonic day 20) (Harris et al., 1989). Approximately a day later they begin to express both the neonatal MHC isoform believed to belong to the fast isoform family and the embryonic MHC isoform, creating the potential for the development of both slow and fast fibre types (Harris et al., 1989; Zhang et al., 1998).

Investigation into the time course of development and innervation of primary and secondary myotubes in rats suggests that 16 days post conception (PC) primary myotubes are heavily poly-innervated (Sheard et al., 1991). Primary myotube innervation declines rapidly 20 - 21 days PC coinciding with a peak in the poly-innervation (multiple neural inputs) of later-formed secondary myotubes. The secondary myotubes then steadily decline in number, through pruning (removal of unrequired motoneurons) to adult levels, beyond day 21 PC (Figure 1.03) (Sheard et al., 1991). As innervation declines, there is a simultaneous change in the expression of surface proteins involved in the attraction of pioneering neurons (the first axons that move through the extracellular matrix (ECM) laying down the initial path, for
Introduction

subsequent axons to pass through (Raper & Mason, 2010)). In particular, there is an increase in the expression of neural cell adhesion molecule (NCAM) on the surface of the primary myotube at 14 - 15 days PC which remains high until birth (22 days PC), before gradually declining over the first two postnatal weeks as pruning of axons occurs (Covault & Sanes, 1986; Sheard 

et al., 1991). This increase coincides with maturation of myoblasts to myofibres (Figure 1.03) (Sheard et al., 1991).

I hypothesise that a decline in NCAM expression correlates with increasing neural activity at the neuromuscular junction (N MJ) associated with the mono-innervation of maturity. Furthermore, increasing activity drives excitation-transcription coupling signals which have an inhibitory influence on NCAM expression thus inhibiting poly-innervation of the mature muscle fibre.

**Figure 1.03 Muscle developmental changes.**
The changing number of nerve inputs to both primary (blue) and secondary (green) myotubes post conception (PC) and the changing level of NCAM expression on the surface of both myotube forms (red). Identified large numbers of neural inputs in both primary and secondary myotubes associated with high levels of NCAM were identified. Both fibre types show declining inputs to adult levels correlating with declining NCAM expression. Adapted and modified from Covault and Sanes (1986), Sheard et al. (1991).
1.3.2 Structure and function

Muscles are formed of long multinucleated fibres containing many thin striated myofibrils. The striations of the myofibrils are formed by repeating units called sarcomeres which are formed of overlapping thin (actin) and thick (myosin) filaments. Thin filaments consist of a tropomyosin, a troponin complex wrapped around an f-actin chain. The thick filaments (myosin) consist of long rod-like structures formed of two sub-fragments, the light and heavy meromyosin (Squire, 1975). Interactions between actin and myosin subunits causes the sarcomere to contract to generate the force that allows muscles to perform functions such as posture maintenance and stability, movement generation, energy storage (glycogen), thermoregulation and roles in metabolic pathway activation (Ohlendieck, 2010).

Skeletal muscles, unlike smooth and cardiac muscle, are composed of slow and fast fibre types that are each characterised by their speed and duration of contractions and by their fatigue resistance. At the molecular level, the characteristics of the fibre types result from the expression of different myosin heavy chain isoforms within the myofibres (MHCI, IC, IIC, IIAC, IIA, IIAB and IIB) (Scott et al., 2001). Specifically, it is the ATPase activity of each MHC group that acts as the major determining factor in the speed of contraction (Zhang et al., 1998). The two basic fibre types are characterised by their different contractile properties (speed of shortening). Fibres expressing MHCIIa/x are large diameter fibres that contract fast, important for maximal power generation/anaerobic activity. MHCI expressing fibres are smaller in diameter and slower contracting, but can maintain active tension important in aerobic activity (Zhang et al., 1998; Mitchell et al., 2012). Skeletal muscles that have fibres of more than one type tend to have the fibre types distributed in a mosaic pattern (random type distribution) across the muscle (Figure 1.07). In contrast, some muscles have more of one fibre type than the other based on the muscle’s function. For example, tibialis anterior is predominantly formed from fast type IIb fibres and lacks slow fibres, allowing fast strong muscle shortening (Piétri-Rouxel et al., 2009).

Skeletal muscle function is modulated by neuronal input from a subset of lower motoneurons alpha motoneurons (α-MN) of the peripheral nervous system (PNS) at the NMJ which determine the activity of the muscle fibres based on their firing pattern. These are two primary firing patterns: A) phasic firing, associated with fast twitch muscle fibres, and B) tonic firing neurons associated with slow twitch muscle fibres.
Neuronal firing stimulates pre-synaptic release of acetylcholine (ACh) leading to post-synaptic ACh receptor binding and depolarisation of the membrane of the muscle fibre at the NMJ. Depolarisation stimulates inward movement of extracellular calcium ions (Ca$^{2+}$) which causes the intracellular release of Ca$^{2+}$ from the sarcoplasmic reticulum, both of which are required for excitation-contraction coupling and force generation (Figure 1.04). A major pathway for extracellular Ca$^{2+}$ entry in skeletal muscle is the voltage sensitive L-type Ca$^{2+}$ channels which are linked to transcriptional activity through cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) activation (Wheeler et al., 2008). The L-type Ca$^{2+}$ channel is composed of five subunits one of which is the dihydropyridine receptor (α1S), a transmembrane protein containing a Ca$^{2+}$ selective pore and voltage sensor loop. α1S is predominantly located at the T-tubules and triads within skeletal muscle where it is associated with the embedded ryanodine receptor (RyR1) of the sarcoplasmic reticulum, which is involved in the intracellular release of Ca$^{2+}$ and excitation-contraction coupling (Piétri-Rouxel et al., 2009). It is this calcium-linked role of α1S that is essential for the regulation of skeletal muscle innervation, protein expression and activity (Piétri-Rouxel et al., 2009); (Bhalla & Iyengar, 1999).

**1.4 Neuromuscular junction**

The NMJ is a cholinergic synapse that transduces electrical information from the cell body of α-MN located in the ventral horn of the spinal cord to the muscles. The NMJ is remarkably stable over time, and the majority of the changes that do occur are not related to ageing (Lichtman & Sanes, 2003), rather the NMJ form and function changes in response to activity and development (Hill et al., 1991; Arnold et al., 2014). The major difference between young and old age involves change in stability of the NMJ (Deschenes, 2011; Li et al., 2011). In senescence the NMJ undergoes morphological changes in the post-synaptic motor-endplate, including fragmentation and formation of receptor islands that can be denervated or poly-innervated (two axons converging onto the same post-synaptic site). Pre-synaptic changes include axon terminals that may be misshapen, swollen or unusually branched (Table 1.01) (Figure 1.05.) (Chai et al., 2011; Gonzalez-Freire et al., 2014).
The extent of neuromuscular dysfunction as a result of ageing has been monitored using acetylcholine receptor (AChR) staining (Li et al., 2011). Degenerative morphological changes accelerate with ageing; an 80% increase in NMJ fragmentation was seen at 22 months compared to 18-month-old mice (Shigemoto et al., 2010; Deschenes, 2011; Li et al., 2011). The nature of the cellular changes that result in destabilisation of the NMJ remain unknown.

**Figure 1.04 Neuromuscular communication**
A single lower motoneuron innervating multiple myofibres (a) within the endplate region of the muscle (b) at the neuromuscular junctions (c). At this point excitation-contraction coupling occurs through the release of synaptic vesicles within the synaptic cleft to induce post-synaptic acetylcholine receptor (AChR) binding and electrical activation of skeletal muscle. Adapted and modified from Conti-Fine et al., 2006.
Table 1.01 Summary of morphological changes in NMJ with age

<table>
<thead>
<tr>
<th>Pre-synaptic</th>
<th>Post-synaptic</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑ complexity of nerve terminal branching</td>
<td>↑ incidence of abandoned synaptic gutters (no AChR)</td>
</tr>
<tr>
<td>↑ nerve terminal branch number</td>
<td>↑ dispersion of acetylcholine receptors</td>
</tr>
<tr>
<td>↑ total length of nerve terminal branches</td>
<td>↓ length of endplate</td>
</tr>
<tr>
<td>↑ area of acetylcholine vesicle clusters</td>
<td>↓ total area of the endplate region</td>
</tr>
<tr>
<td>↓ total number of acetylcholine vesicles</td>
<td>↓ total perimeter length of the endplate region</td>
</tr>
<tr>
<td></td>
<td>↑ incidence of pre- to post synaptic uncoupling</td>
</tr>
</tbody>
</table>

Adapted and modified from (Deschenes, 2011)
Investigations assessing temporal changes in NMJ remodelling and the concurrent innervation status of the junctions suggest that changes in the denervation/re-innervation process begin at the NMJ and progress in a retrograde fashion back to the cell body in the spinal cord (Deschenes, 2011). This suggests morphological changes with age may be a result of shifts in expression of maintenance substances at the NMJ. Therefore, age-related changes in muscle structure and function may be a secondary consequence of degenerative changes at the NMJ.

To investigate the loss of NMJ stability that may drive age-related denervation, the Sheard laboratory previously investigated five candidate NMJ maintenance proteins: Low density lipoprotein receptor-related protein 4 (Lrp4), Rapsyn, Agrin, Docking protein 7 (Dok7) and Muscle-specific receptor tyrosine kinase (Musk) (Bruneau & Akaaboune, 2010; Shi et al., 2012; Wu et al., 2012; Zong et al., 2012). Rapsyn, Agrin and Musk proteins showed correlative declines in expression with morphological indices of sarcopenia (Gillon, 2013). This rate of decline correlates with the neuromuscular
dysfunction associated with sarcopenia, which posits that loss of NMJ maintenance proteins are key factors in muscle denervation. How these changes relate to a muscle’s subsequent inability to attract a nerve terminal sprout have yet to be elucidated.

1.5 The peripheral nervous system

The peripheral nervous system consists of both motor and sensory branches that extend from the spinal cord or brain to their target organ. The motor branches are the focus of my work as they drive skeletal muscle fibres. In a healthy mouse the relationship between the motoneuron and muscle fibre undergoes ongoing modification as a physiological part of neuronal maintenance associated with changing activity patterns of the neuromuscular system (Deschenes, 2011). However, in ageing changes in neuronal input to skeletal muscle fibres occurs.

1.6 Age-driven denervation

The multifaceted manifestation of sarcopenia makes discerning a temporal relationship between the changes at the muscular level and the α-MN level difficult, but data support the following generalised schema for age-related neuromuscular changes. Motoneurons driving large, fast motor units (MU) seem differentially susceptible to premature death, and these neurons die early due to unknown causes (Campbell et al., 1973; Tomlinson & Irving, 1977; Lexell, 1997; Cruz-Sanchez et al., 1998; McNeil et al., 2005). Their death leaves seemingly healthy fast muscle fibres denervated, and the response of denervated fibres is normally to emit a “denervation signal” which initiates sprouting from nearby intact motor nerves or nerve terminals. Sprouts grow to re-innervate denervated fibres with several structural and functional consequences. First, surviving motor units tend to enlarge to incorporate a larger number of muscle fibres. Second, as surviving α-MNs are increasingly associated with “slow” muscle fibres (express MHCl) there is an increasing tendency for re-innervated fibres to undergo transformation from fast (express MHCIIa, IIx/d or IIb) to slow types, and for slow fibre type clumping, (grouping of like fibre types together within the muscle) to occur. Several observations support the existence of each of these events (Lexell et al., 1986; Lexell & Downham, 1991; Evans & Lexell, 1995; Deschenes, 2011), and data from our own lab are consistent with these events being at least partially correct (Figure 1.06) (Deschenes, 2011; Mitchell et al., 2012). However, there is also evidence that some denervated fibres fail to become re-innervated by
neighbouring α-MNs, resulting in long-term denervation which has been shown to lead to muscle fibre atrophy (Viguie et al., 1997; Rowan et al., 2012).

It has been proposed that an increase in MU size with decreasing numbers of neurons results in a declining ability to re-innervate (Tam & Gordon, 2003a). However, investigations into MU branching pattern and efficacy have established that each MU can enlarge five to eight-fold (Brown et al., 1981). Failure to re-innervate and subsequent weakness become evident only when less than 20% of the functional MUs remain (Son & Thompson, 1995; Luff, 1998; Tam & Gordon, 2003a). If the capacity for neurons to re-innervate neighbouring denervated muscles is retained with age, this poses the question of what alterations are causing the permanent denervation associated with atrophying fibres. One hypothesis is that absolute loss of α-MNs with age results in a decreased capacity for neuronal sprouting following denervation (Campbell et al., 1973; Tomlinson & Irving, 1977; Ishihara & Araki, 1988; Valdez et al., 2010; Rowan et al., 2012). The cause of α-MN death remains controversial despite many studies investigating age-related changes in neuron cell body number within the lumbosacral portion of the spinal cord. In rats, Rowan et al. (2012) showed a 27% decrease in the number of lumbar spinal cord motoneurons between young adult (8 - 10 month) and senescent (33 month) rats. Similarly, Tomlinson and Irving (1977) demonstrated that in humans there is no significant change in cell number until age 60, after which a negative correlation between cell number and patient age was observed. By age 90, a 70% decrease in α-MNs
was noted compared to subjects at age 60, suggesting a rapid age-related decline in muscle innervation (Covault & Sanes, 1985, 1986; Andersson et al., 1993; Deschenes & Wilson, 2003). In contrast, some studies have found that only a 10 - 15% loss of motoneurons occurs with age in mice (Edstrom et al., 2007), while further studies in mice have found no loss of lower α-MNs but noted numerous degenerative changes at the level of the NMJ (Chai et al., 2011). In summary, characteristic changes in NMJ morphology appear to precede denervation and the presence of expanded motor units and evidence of motor nerve terminal sprouts shows that at least some denervated fibres seem capable of re-innervation. This suggests that atrophy of muscle fibres associated with sarcopenia results from changes not solely attributable to α-MN death (Deschenes et al., 2010).

1.7 Response to axonal injury

The peripheral nervous system adapts following injury with a regenerative process (see below) involving the motor endplate, nerve terminal (pre-synaptic terminal) and Schwann cells (Liu et al., 2011). The response of the PNS to axotomy (cutting or severing of an axon) is split into three phases.

Phase 1: Acute Axonal Degeneration (15 minutes post injury).

Acute Axonal Degeneration (AAD) occurs in response to the initial lesion. Cytokines including histamine and serotonin, stimulate inflammation at the site of injury which initiates macrophage infiltration. The onset of the lesion also causes damage to surrounding vasculature which results in cavitation, the uncontrolled movement of fluid from the vasculature into the surrounding tissues. This movement of fluid forms a fluid-filled space that demarcates the secondary damage zone surrounding the initial lesion site (Glasby et al., 1997). The onset of injury is also associated with surrounding axonal Schwann cells undergoing both nuclear and cytoplasmic enlargement and increasing mitotic rates resulting in rapid divisions into dedifferentiated daughter cells (Burnett & Zager, 2004), which are associated with further degenerative and repairing processes, for example the acute degeneration of the proximal and distal ends of the severed axon (up to 50 microns) (Wang et al., 2012). The degeneration of the axonal segments occurs in response to a large increase in intracellular sodium (Na+) through the lesion site. Na+ influx triggers action potential generation, and is a signal for the influx of extracellular Ca2+ through voltage-gated channels and release of Ca2+ from intracellular stores (endoplasmic reticulum and mitochondria). The large influx of cytosolic Ca2+ stimulates
Ca\textsuperscript{2+}- dependent activation of the serine-threonine protease calpain, which in turn stimulates rapid degeneration of axonal segments through cleavage of axonal neurofilament and microtubule associated components (Johnson et al., 1991). Calpain induced degeneration of the axon is thought to stimulate necrotic signals that attract phagocyte and macrophage infiltration. The early onset degeneration is hypothesised to generate an optimised environment for regeneration of the proximal axon following lesion (Figure 1.07B) (Wang et al., 2012).

**Phase 2: Latency period**

The latency period (24 - 48 hours post injury) is characterised by the formation of a dystrophic cone on the end of the axonal segments followed by 24 hours of structural quiescence. Dystrophic cone formation prevents further Na\textsuperscript{+} influx through the severed ends, inhibiting further calpain initiated degeneration. During the latency phase the axonal lesion prevents retrograde and anterograde signalling through neurotrophic factors between the NMJ and cell body. Along with increased action potential firing and cytosolic Ca\textsuperscript{2+}, the decreased neurotrophic signal stimulates chromatolysis (the dissolution of the neuronal nissl bodies). The nucleus of the neuron migrates to a peripheral position in the soma, at which point the chromatin within the nucleus begins to degenerate (He & Koprivica, 2004). This process signals perineuronal glial cell processes to isolate the affected neuron and interrupt signalling in preparation for the regenerative phase (Burnett & Zager, 2004). Prolonged denervation results in signals to upstream synapses that the axon is dying and stimulates proximal transneuronal synaptic retraction (Figure 1.07C) (He & Koprivica, 2004).

**Phase 3: Wallerian degeneration**

The Wallerian degeneration phase (weeks-months post injury) is characterised by rapid (24 mm/hr in C57/B6j mice) fragmentation and cytoskeletal breakdown along the full length of distal axons (Sievers et al., 2003). Wallerian degeneration is initiated in response to the cessation of anterograde and retrograde signalling in the distal axon, provoking retrograde or anterograde degeneration of the distal segment depending on injury site, leaving behind only a collapsed column of surrounding Schwann cells forming a structure known as the bands of Büngner (von Büngner, 1890; Burnett & Zager, 2004; Wang et al., 2012). The regenerative proximal axon can then use the bands of Büngner as guides towards the denervated muscle. The regenerative process begins
as the neuronal nucleus is relocated centrally where Nissl granules are reorganised for axoplasm generation. Sustained protein and lipid generation coupled with axonal transport of proteins and axoplasm to the proximal tip drive the regenerating axon (Figure 1.07D/E).
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Figure 1.07 Peripheral Nervous System response to axotomy

A) The intact neuron and axonal Schwann cells connect with skeletal muscle via the NMJ. B) The acute axonal degeneration following axotomy involves Na⁺ influx stimulating Ca²⁺ intracellular release and calpain activation. The resultant effects are large reductions in both distal and proximal neuron length as a result of degradation of neurofilament proteins. C) The latency period is characterised by chromatolysis and a halt in structural modifications to axonal framework. D) Wallerian degeneration includes degeneration of the distal axon, de-clustering of the AChR on the muscle surface and the beginnings of the regenerative process. E) Regeneration involves axolemmal protein transport to the protruding axon tip. Growth is guided by interaction with chemotactic markers in the ECM and cell adhesion molecules expressed on the surface of de-differentiated Schwann cells. F) The regenerated axon re-contacts the denervated muscle, stimulating re-clustering of AChR and reformation of the NMJ. G) Sarcopenia, the loss of ability to re-innervate, occurs as a result of changes in expression of “come get me” factor on the muscle surface. Prolonged denervation results in trans-neuronal denervation of upstream axons and permanent denervation of the muscle. 

Abbreviations: Rough endoplasmic reticulum (RER), Acetylcholine receptor (AChR), Extracellular matrix (ECM), Neuromuscular junction (NMJ) neurite attracting factor (NAF).
1.8 Axonal sprouting

Axonal sprouting is the protrusion of axolemmal proteins from the parent axon towards the target site. The process delineates the early stages of re-innervating, and is similar to the formation of the growth cone during development (Ide, 1996). Sprouts originate from one of three sites in response to a re-innervation stimulus; ultraterminal sprouts from the motor endplate region, pre-terminal sprouts from the nerve terminal region and nodal sprouts from the nodes of Ranvier (Brown et al., 1981; Hopkins & Slack, 1981; Tam & Gordon, 2003a). One of the key mechanisms required in response to denervation, and in the absence of bands of Büngner, are terminal Schwann cells (tScs), which form extensive bridging processes. Terminal Schwann cell processes branch away from both denervated and innervated endplate regions terminating at the abandoned muscle fibres, therein actively guiding re-innervation through new formation of bands of Büngner (Tam & Gordon, 2003a). Terminal Schwann cell bridging is an activity-dependent process driven by tSc membrane expression of muscarinic acetylcholine receptors (mACHR) that initiate Ca$^{2+}$ influx in response to pre-synaptic release of acetylcholine (Rodnight et al., 1997; Love & Thompson, 1998). Activity-dependent mACHR activation has been correlated with the prevention of tSc branching through glial fibrillary acidic protein (GFAP) inhibition. Once skeletal muscle becomes denervated, neuromuscular signalling is silenced. Silenced signalling reduces Ca$^{2+}$ influx to the Schwann cell disinhibiting GFAP synthesis. Ultimately an up-regulation GFAP synthesis promotes bridging of innervated and denervated endplates (Figure 1.08) (Rochon et al., 2001; Tam & Gordon, 2003b). Bridging enables neurite outgrowth by facilitating guidance of new axonal sprouts to the abandoned muscle fibres via chemotrophic interactions with the surrounding environment. The formation of the bridge generates a scaffold for the growing sprout. Extension of the sprout within the column is essential as the surrounding connective tissue inhibits extension and regeneration. Successful extension within the column requires attachment of the growing axon to the inner surface of the bands of Büngner (Ide, 1996).

Axon sprouting is mediated through a series of cell adhesion molecules including NCAM, L1 and P0, all of which are essential for pioneering neurons during development (Rutishauser, 1985). These molecules are up-regulated following denervation at the tip of the growing axon and in the Schwann cells that form the bridge. Expression of these cell adhesion molecules is thought to promote growth of the axon along the column.
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(Figure 1.07E). The extension of the neurite though mediated by interactions with the surrounding Bungner wall is catalysed by protein kinase C (PKC) intracellular signalling, which is highly active in the growth of regenerating axons. PKC activity is important in gene transcription essential for the protein production and axonal transport required to extend the growth cone towards the denervated muscle (Kawano et al., 1997). This ability to regenerate following axotomy is specific to the PNS as a series of inhibitory factors prevent the process occurring in neurons of the central nervous system (CNS)(Yiu & He, 2006). The regenerative capacity is also vital in establishing and re-establishing connections that underlie the active neuromuscular system. However, if this system and regenerative capacity are altered or lost with age, this could explain the inability of elderly muscles to become re-innervated.

(Figure 1.08) Activity dependent mediation of terminal Schwann cell (tSc) bridge formation following skeletal muscle denervation. (A) In a healthy neuromuscular junction (NMJ) an action potential travels down the axon (1) stimulating depolarisation of the presynaptic terminal and release of synaptic vesicle contents (2) into the synaptic cleft. Acetylcholine (ACh) travels across the synaptic cleft and binds to nicotinic acetylcholine receptors (nAChR) on the post-synaptic muscle fibre. ACh binding to nAChR causes calcium influx (3) and stimulates skeletal muscle depolarisation and contraction (4). ACh also binds to the muscarinic acetylcholine receptor mAChR on tSc (5) stimulating calcium influx (6). This influx inhibits glial fibrillary acidic protein (GFAP) synthesis and prevents bridging. (B) The denervated muscle fibre prevents action potential formation within the axon (1). Absence of action potentials inhibits synaptic vesicle cycling release (2) and ultimately the influx of calcium in both muscle (3) and tSc (4 & 5). The inhibition of tSc calcium influx disinhibits GFAP synthesis which stimulates up-regulation and activates tSc bridging towards the abandoned muscle fibre (6).
1.9 The ageing peripheral nervous system

The PNS is susceptible to age-related alterations including alterations to normal functioning of the neuronal synapse, changes in neurotransmitters, energy production and gene transcription (Esiri, 2007). The PNS also undergoes reduction in conduction velocity and becomes susceptible to degradation of the myelin sheath, the insulating structure of peripheral axons. Reduction in myelin is likely linked to the decline in both sensory and motor neuron conduction velocity seen during ageing (Verdú et al., 2000). Human neurons exhibit a steady decline in conduction velocity of both sensory (64.3 to 56.9 ms⁻¹) and motor (59.4 ms⁻¹ to 52.6 ms⁻¹) portions of the median nerve from age 18 to 60-86 years respectively (Dorfman & Bosley, 1979; Verdú et al., 2000), suggesting significant reductions in transmission between the brain and muscle. This altered conduction velocity conveys a rostral-caudal variance that correlates with varied susceptibility to muscle fibre atrophy within the rostral- (upper limb) caudal- (lower limb) axis (Sheard & Anderson, 2012).

The hypothesis currently under investigation in our lab for this differing rostral-caudal susceptibility suggests an age-related reduction in anterograde and retrograde neural transport capabilities as a function of increased axonal length. With increasing length from the cell body there is increasing molecular turnover, resulting in a reduction of total neurotrophic factor that reaches the muscle. Since lower limbs have proportionately longer axons an increased turnover coincides with differences seen between muscle losses in the upper and lower limb (Unpublished Brady J). Proteins within the neuron that are trafficked include trophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 4 (NT4) and neuregulin (NRG), all of which are essential for both neuron and muscle survival (Delbono, 2003; Omura et al., 2005; Cui, 2006; Gumà, 2010).

One of the major alterations to PNS function is the response of neurons to axotomy or surgical denervation of muscle with progressive ageing. A large majority of investigations have documented comparable regenerative abilities between young and aged animals, but indicated a temporal delay and in some cases a reduction in the regenerative capabilities of aged animals (Figure 1.09.) (Pestronk et al., 1980; Vaughan, 1992; Verdú et al., 2000). This posits that although slowed, the aged α-MNs still possess the ability to re-innervate denervated muscle. This raises the question of whether a
neurite attracting factor (NAF) required to initiate the re-innervative process is lost with age, thereby preventing successful re-innervation.

The stimulus that promotes neurite outgrowth following denervation is yet to be identified but is thought to be a short range diffusible factor. The effective range of the diffusible factor was evaluated by determining the ability of slow and fast $\alpha$-MNs to re-innervate muscle fibres following partial denervation. Denervated fibres were re-innervated by sprouts from terminals of neighbouring neurons up to 200 $\mu$m from the muscle. However, these distances were greater when measured from the endplate region suggesting that both the extra-junctional and endplate zone release NAF in response to denervation (Pockett & Slack, 1982).
The short range of such a diffusible factor potentially underpins the fibre type clumping phenomenon, as it allows a single $\alpha$-MN to innervate denervated muscle fibres within a close proximity to itself (Figure 1.10) (Cornwall & Sheard, 2011). This, combined with the ageing nervous system retaining its innate ability to re-innervate and sprout, indicates that the framework to re-innervate is still intact. During sarcopenia I theorise, permanent denervation and resulting fibre atrophy are consequences of age-associated alterations to the production of a post-synaptic (muscular) diffusible factor(s) which normally facilitate re-innervation.

**Figure 1.10 Short range diffusible factor**
The presence of a short-range diffusible factor would create a pattern in which a centralised fibre (red) would have the ability to re-innervate a series of fibres surrounding it (A). If those fibres became denervated (blue) (B), they would be re-innervated by the $\alpha$-MN of the centralised muscle fibre creating what appears as a clumping of fibre types (red) (C).
1.10 Neural cell adhesion molecule

Neural cell adhesion molecule (NCAM) is a protein synthesised from the NCAM1 gene on the 11th chromosome in humans and a homologous position on the 9th chromosome in mice (Walsh & Doherty, 1991). From this single gene three isoforms with three distinctive molecular weights are synthesised; 180 kilodaltons (kDa), 140 kDa (difference based on the length of the cytoplasmic domain) and 120 kDa (Covault et al., 1986; Walsh & Doherty, 1991; Walsh et al., 2000; Panicker et al., 2003). All three isoforms have a generic ECM domain consisting of five immunoglobulin (Ig) domains of 7 and 9 antiparallel β-strands arranged in two β-sheets (Bork et al., 1994), and two fibronectin 3 (FNIII) domains (Figure 1.11) (Walsh & Doherty, 1997).

![Figure 1.11 The isoforms of NCAM](image)

The three forms of NCAM, the transmembrane linked form (180 and 140 kDa), the GPI linked form (120 kDa). Adapted from Walsh & Doherty, 1991.
Introduction

These ECM domains are essential in both the cell adhesion and signalling roles of NCAM. Despite the structural similarities of the ECM domains, the three isoforms of NCAM differ in both their presence of a transmembrane spanning domain and their primary location of expression. The 180 kDa isoform consists of a transmembrane spanning domain and is predominantly expressed at the pre-synaptic membrane. The 140 kDa isoform, which possesses a transmembrane spanning domain and the 120 kDa isoform, which has a glycoprophatidylinositol (GPI) membrane anchor, are expressed on neurons, Schwann cells, astrocytes and skeletal muscle (Edelman, 1983; Rutishauser, 1985; Covault et al., 1986).

The activity of NCAM is modulated by a long chain carbohydrate, polysialic acid (PSA), specifically a homopolymer of α2,8 sialic acid. PSA is expressed within the brain during development and has a key role in neural morphogenesis and neurite elongation. PSA localises to NCAM via a glycosylation post translational modification, in which PSA is added to the fifth Ig domain of any of the NCAM isoforms (Seki & Arai, 1993; Rønn et al., 2000). PSA is synthesised on NCAM through the presence of either polysialic transferase ST8Sia IV (PST) or ST8Sia II (STX), which are highly homologous and share 59% identity (Angata & Fukuda, 2003). PSA chains consist of up to 200 sialic residues resulting in a molecular weight of 60 kDa (Angata & Fukuda, 2003). Both the size and conformation generate a large negative charge which, once bound, results in steric hindrance of NCAMs homophilic binding properties.

NCAM mouse knockout model (KO) investigations have shown no lethality, however major changes in the brain’s architecture have been identified, particularly in highly plastic areas such as the hippocampus and olfactory bulb. Changes include reductions in axonal outgrowth, reduced long term potentiation (LTP), and deficits in spatial memory tasks identified via the Morris water maze task (Murase & Schuman, 1999; Paratcha et al., 2003). It has also been noted that a reduction in the numbers of projections from the retinotectal area of the brain requiring long axon growth during development to reach their synaptic sites are seen to persist in NCAM-deficient models ( Walsh & Doherty, 1997; Rønn et al., 2000; Angata & Fukuda, 2003). Along with KO models for NCAM, the importance of PSA and polysialylated NCAM has been investigated by enzymatically cleaving polysialic acid from NCAM using neurominidase. The addition of neurominidase to in vitro cell cultures and in vivo brain areas resulted in decreased plasticity and inhibition of neurite outgrowth (Rønn et al., 2000). Similar alterations to neuronal signalling and neurite outgrowth occur when PSA and NCAM are removed,
which implies the presence of PSA and NCAM are vital in the development of brain and skeletal muscle.

The actions of NCAM are made possible by interaction with both the surrounding ECM environment and other surrounding cells. Initially NCAM was thought to act only as a cell adhesion molecule through homophilic binding (NCAM–NCAM) (Rønn et al., 2000; Rønn et al., 2002). The homophilic binding interaction is proposed to occur at an interface between the third Ig domains of each NCAM. More recently it has been suggested to involve a double zipper method in which interaction between the first and second Ig domains of each NCAM molecule occurs when the amino acid chains are in the trans conformation (Walmod et al., 2004; Kiselyov et al., 2005). Further investigation however has revealed that NCAM has additional heterophilic binding to a series of ECM proteins including heparin sulphate and chondroitin sulphate proteoglycan. This binding is thought to be vital for the stimulation of neurite outgrowth and fasciculation, the tendency for neurons to grow along a pioneered path forming the nerve fascicle (Rutishauser et al., 1983; Olsen et al., 1993). Along with innate cell adhesion properties, NCAM also possesses an intracellular signalling mechanism through interaction with fibroblast growth factor receptor (FGFR). Activation of FGFR drives intracellular signalling through phospholipase C gamma (PLCγ), culminating in Ca^{2+} influx from intracellular stores (Figure 1.12) and activation of PKC which drives neuronal outgrowth and synaptogenesis during development. Protein kinase C activation is also an essential part of the re-innervative process previously mentioned to occur in the PNS (Bassel-Duby & Olson, 2006). Regulation of intracellular signalling properties has been closely linked to the presence or absence of PSA. The aforementioned steric hindrance of PSA inhibits NCAMs cell adhesion properties and promotes intracellular signalling, ultimately stimulating neurite outgrowth (Walsh & Doherty, 1991).
Schematic of intracellular signaling pathways activated following polysialylated NCAM binding. The major pathway involves phosphorylation of Fibroblast Growth Factor Receptor (FGF-R) by polysialylated NCAM (PSA-NCAM) promoting interaction with phospholipase C gamma (PLCγ). Activation of PLCγ catalyses the activation of the phosphatidylinositol phospholipase C (PI-PLC) which initiates two vital pathways. The cleavage of phosphatidylinositol 4,5-bisphosphate (PIP₂) into the second messengers inositol 1,4,5-trisphosphate (IP₃), and diacylglycerol (DAG). Each second messenger then targets their downstream pathways; DAG acts either to activate protein kinase C (PKC), initiating the mitogen associated protein (MAP/MEK/ERK1/2) pathway of gene transcription, or is converted by DAG lipase to 2-arachidonoylglycerol (2-AG) and arachidonic acid (AA) in the initiation of cell calcium (Ca²⁺) permeabilisation. IP₃ associates with rough endoplasmic reticulum (RER) bound Ca²⁺ channels resulting in the release of intracellular Ca²⁺. Together, the increased permeabilisation and intracellular release of Ca²⁺ increases cytoplasmic Ca²⁺ concentration. Increased Ca²⁺ activates both Calmodulin kinase 2 (CamKII) and adenylyl cyclase, two pathways that act to promote CREB activation and gene transcription via cyclic Adenosine MonoPhosphate (cAMP). Ultimately, the aforementioned pathways modulate neuronal activity to promote neurite outgrowth and fasciculation. PI-PLC acts to cleave the GPI bound 120 kDa isoform from the membrane releasing it as a soluble peptide into the ECM. Adapted and modified from Walmad et al., 2004.
During embryonic development, high NCAM expression is believed to be essential for the proper development of synaptic connections in both the brain and skeletal muscle. In conjunction with high NCAM, high levels of PSA drive polysialylated NCAM formation to encourage the neural plasticity, outgrowth and fasciculation required for synaptogenesis (Walsh & Doherty, 1991). This early form of NCAM (NCAM-E) is associated with NCAM presence during embryogenesis as an important attractor of neural input (Grumet et al., 1982; Covault et al., 1986). During late development, levels of NCAM decrease in all regions to below the threshold required to stimulate neurite outgrowth (Figure 1.13). At levels below this threshold NCAM-E is believed to transition to adult NCAM (NCAM-A) which is thought to maintain the synapse acting as a cell-adhesion molecule (Walsh & Doherty, 1991; Martini, 1994). Exceptions to this are seen in the hippocampus, dentate gyrus and olfactory bulb which maintain high levels of NCAM-E with age (Angata & Fukuda, 2003).

![Figure 1.13 NCAM activity threshold](image)

The relative amount of NCAM present in a cell compared to the stimulated neurite outgrowth. In contrast to N-cadherin, another cell adhesion molecule, it appears that NCAM requires a threshold level of synthesis before it can stimulate neurite outgrowth. Adapted and modified from Walsh & Doherty, 1991.

These maintained levels of NCAM may be required for high plasticity with continued pruning, axon outgrowth and high levels of new growth in these areas to maintain memories and olfaction (Rousselot et al., 1995). Therefore, it has been suggested that NCAM expression is activity dependent and driven by neural input (Angata & Fukuda, 2003). During synapse formation, NCAM and PSA levels are high with relatively low activity favouring NCAM signaling. Once formed, the levels of NCAM and PSA decrease thus increasing synaptic activity to stimulate NCAM cell adhesion roles for synapse
maintenance. NCAM is not restricted to the formation of the synapse; expression on Schwann cells and the pioneering neurons during development are essential for guidance through the ECM. In a similar manner to muscle during maturity, NCAM is down-regulated on Schwann cells (Martini, 1994) and a change in NCAM expression correlates with denervation, termed denervation stimulated up-regulation. Up-regulation of NCAM on both the Schwann cell surfaces and axons is thought to be part of the re-innervative process whereby NCAM mediates passage of the regenerating sprout along the bands of Böngner (Martini & Schachner, 1988). It is suggested that this up-regulation on the growing axon and the bands of Böngner promotes outgrowth directed towards the denervated muscle.

Further investigation of the structure and mechanism of NCAM function has identified that in addition to the three membrane-bound isoforms, NCAM can become spontaneously cleaved to a soluble form (Olsen et al., 1993; Secher, 2010). The cleavage of a soluble peptide (Secher, 2010) involves activation of the phosphatidylinositol-specific phospholipase C (PI-PLC) pathway which cleaves the 120 kDa isoform of NCAM from the membrane. The cleavage of NCAM is linked to the activation of PSA-NCAM intracellular signalling, suggesting that up-regulation of NCAM and PSA stimulate the release of soluble NCAM (Figure 1.12). The released form is expressed as a truncated (110 kDa) form of the GPI-bound NCAM isoforms (Secher, 2010). Despite cleavage, the truncated isoform maintains the active domains and binds transmembrane bound NCAM to activate intracellular signalling pathways in vitro (Olsen et al., 1993). Based on both its presence throughout the ECM and its ability to activate transmembrane bound NCAM it has been suggested that release of soluble NCAM can act as a chemotrophic factor, involved in neurite growth and more importantly guidance during sprouting and potentially re-innervation (Olsen et al., 1993). A soluble “come get me” factor is required following skeletal muscle denervation to stimulate re-innervation, therefore soluble NCAM could potentially serve this purpose.

Although a large amount of work has been carried out to characterise the structure, function and interactions of NCAM during development and maturity, the changes that occur with age, particularly at the level of skeletal muscle, have yet to be examined. Research has shown that during development there are high levels of NCAM-E, both junctionally and extra-junctionally, involved in the formation of the NMJ through neurite growth followed by cell adhesion (Covault & Sanes, 1985). During late development
however, there is a down-regulation of NCAM-E as it transitions from concentrated to
sparse localisation, exclusively at the junctional folds of the NMJ (NCAM-A) (Covault &
Sanes, 1985). Concurrently, down regulation in the amount of PSA-NCAM present in
skeletal muscle has been observed (Olsen et al., 1995). These two changes are consistent
with the hypothesis that during development large amounts of PSA-NCAM-E are
required to promote neurite outgrowth and NMJ synapse formation, whereas during late
development NCAM transitions to NCAM-A with down-regulated PSA expression that
promotes cell adhesion and synapse modulation.

This down-regulation is temporary as investigations have shown that in mature and
aged mice (24 months) skeletal muscle denervation stimulates an up-regulation of
NCAM at both the junctional and extra-junctional locations. Using both mRNA and
immunohistochemical quantification, NCAM appears to exhibit a tenfold increase in
content/expression 10 days after denervation, remaining up-regulated by a threefold
increase for up to 300 days with permanent denervation (Covault & Sanes, 1985, 1986;
Andersson et al., 1993; Deschenes & Wilson, 2003). Similar trends of increased NCAM
expression were seen in response to muscle unloading where the hind limb of the
animal is suspended preventing load bearing by a sub-set of muscle in young and more
pronounced in elderly mouse muscle (Deschenes & Wilson, 2003). Therefore an activity-
dependent change is shared by PSA expression within muscle which is also up-regulated
following denervation. Interestingly, the levels of PSA when re-expressed are not as high
as those present during development (30% of total weight of NCAM to 10% of weight of
NCAM) (Olsen et al., 1995). Such a change reflects an innate ability of skeletal muscle to
revert to its developmental state, allowing the re-expression of NCAM and PSA in an
effort to re-attract a sprout from a nearby nerve terminal or nodal axon.

1.11 Summary

In summary, young denervated muscle fibres express NCAM at high levels seemingly as
part of a mechanism to attract neural input. NCAM is then down-regulated to
maintenance levels once innervation is achieved. In the elderly, many denervated
muscles do not become re-innervated, but it is unknown if this is because the fibres lack
the ability to express increased levels of NCAM, because the nerve has lost the ability to
respond to NCAM, or for some unknown reason. Investigating whether the
neuromuscular system responds to fibre denervation with an effective NCAM-mediated
regenerative response which is reduced with age is the main aim of this investigation.
1.12 Statement of problem

Research within the field of sarcopenia has identified many factors that can contribute to atrophy and loss of muscle fibres with age. However, the cause of permanent muscle fibre denervation remains an important unresolved issue. The apparent loss of the innate ability to re-attract neural input following muscle fibre denervation is of particular interest. Data suggest that denervation is a key contributor to changes in the neuromuscular junction phenotype seen in elderly animals. Motor nerves in young C57Bl/6j mice have the ability to re-innervate muscle fibres following experimental denervation, either as a process of re-innervation from the parent neuron or sprouting from neighbouring neurons in response to an unknown stimulus (Covault et al., 1986). During development, it seems, the naïve muscle expresses an unknown “neurite attracting factor” (NAF) with the apparent aim of attracting a pioneering neurite in the area (Figure 1.14A). Once the muscle reaches its mature level of innervation, the levels of NAF are down-regulated to a sub-threshold level which no longer attracts neurites. This has the added benefits of preventing poly-innervation and maintaining synaptic viability (Figure 1.14B/D). The level of NAF expression is thought to be maintained at low levels throughout life, but the existence of permanently denervated fibres in old muscles suggests that the mechanism fails at some stage. In young animals it is thought that surgical denervation drives up-regulation of the NAF, thus enhancing a muscle fibre’s ability to re-attract innervation by sprouting neighbouring or regenerating motor axons (Figure 1.14C) (Covault & Sanes, 1985). Age-related loss of the ability to re-express the NAF may result in an impaired capacity to induce a restoration of neural input on denervation, and may therefore contribute to long-term denervation in old age (Figure 1.14E/F).

This loss of re-innervative ability raises two questions. First, does elderly muscle possess the ability to re-express this NAF following denervation? Second, if a muscle fibre can re-express a NAF, are nearby motor nerves capable of responding? Investigation of proteins and molecules that fulfil these properties have identified a number of candidates including nicotinic acetylcholine receptor subunits gamma and delta (nAChRγ & nAChRδ). These subunits are important during development and are up-regulated when innervation is lost (Adams et al., 1995). Other candidates include Runt-related transcription factor (Runx-1) which is up-regulated in age as a protective mechanism against muscle fibre atrophy (Wang et al., 2005; Barns et al., 2014), and
myogenin which is a gene transcription factor involved in muscle protein biosynthesis at the NMJ. Motor axon denervation stimulates up-regulation of myogenin gene expression, increasing production of proteins such as nAChR at the NMJ, a receptor essential in aiding synaptic signalling. Myogenin is also up-regulated following both surgical denervation and age (Kostrominova et al., 2000; Moresi et al., 2010). Another influential protein is insulin-like-growth factor-1 receptor (IGF-1R), a molecule essential in muscle fibre anabolic and catabolic signalling pathways (McMahon et al., 2011; Schiaffino & Mammucari, 2011) which is up-regulated between 15 and 24 months in C57Bl/6j mice (Barns et al., 2014). Although each of the aforementioned molecules contribute to the regulation of one or more of the vital steps, I reason that the NAF has to be diffusible to act at a distance, therefore it cannot be membrane bound as these candidates are. Therefore one final candidate protein stands out. NCAM has influential roles in development and maintenance of the synapse (NMJ), re-innervation of the synapse following surgical denervation and has been shown to be diffusible within the ECM. The current aims and hypotheses are based on NCAM and its roles following denervation and in response to ageing.
**Development:** During development, high levels of membrane-expressed and soluble factor are synthesised to stimulate innervation of the muscle fibre by a motor neuron.

**Youth:** Once innervated, the levels of the factor are down-regulated to a sub-threshold level which prevents further innervation, and acts to maintain the terminal.

**Surgical denervation in youth:** Once the muscle fibre is denervated, the factor is up-regulated on the muscle surface and released as a soluble NAF. Stimulates neighbouring neurons to innervate the muscle fibre.

**Adulthood:** The innervated muscle maintains low levels of the factor which is essential for maintenance of the synapse with the neuron.

**Surgical denervation in elderly:** Once the muscle fibre is denervated, the NAF is not expressed on the muscle surface or released as soluble NAF, which prevents neighbouring neurons receiving the re-innervation signal.

**Sarcopenia:** The muscle fibre becomes naturally denervated as a result of age. NAF is not up-regulated on the muscle surface or released as a soluble NAF, so neighbouring neurons do not receive the signal to re-innervate.

**Figure 1.14 The "neurite attracting factor hypothesis"**

Muscle fibres change their innate ability to re-attract a sprout. This ability is present in young C57B6/J animals, but a loss is hypothesised in ageing resulting in a phenotype characteristic of sarcopenia. (A) Development of the neuromuscular junction (NMJ), (B) regulation of the NMJ during life, (C) surgical denervation causes factor up-regulation, (D) regulation during adulthood, (E/F) surgical denervation and sarcopenia alter innervative capacity in aged muscle fibres, neurite attracting factor (NAF).
1.13 Aims and hypotheses

**Aim One:** To quantify changes in NCAM level at surgically denervated NMJs in young mice.

My first aim is to determine whether previously described changes in NCAM expression induced by surgical intervention are replicated in our mice, in my hands.

**Hypothesis:**
That denervated young mouse skeletal muscle fibres will exhibit increases in amount of NCAM.

**Aim Two:** To quantify changes in NCAM level at surgically denervated NMJs in elderly mice.

I will compare the levels of NCAM in muscle tissue from young healthy and surgically denervated mice to those on healthy and denervated elderly mice. I wish to know whether elderly muscle fibres respond to denervation with the same up-regulation of NCAM expression that is a feature of denervated young skeletal muscles.

**Hypothesis:**
That the surgically denervated elderly muscles will show impaired up-regulation of NCAM expression compared to the young denervated animals.

**Aim Three:** To compare polysialylated and un-polysialylated NCAM levels at the neuromuscular junction following denervation.

The third aim is to investigate changes in the relative proportions of NCAM proteins that have undergone post-translational modification (PTM) specifically by the addition of polysialic acid (PSA). This will enable me to quantify the proportion of active versus inactive NCAM expression at the NMJ following surgical denervation in young and in elderly animals affected by sarcopenia.

**Hypothesis:**
That the expression levels of both PTM NCAM (PSA-NCAM) and unNCAM will be representative of the roles that each isoform has in the re-innervative process. In particular, PSA-NCAM will show a greater increase in expression in surgically denervated young mice but not in elderly mice.
Introduction
2. CHAPTER TWO
METHODS
2.1 Overview

I aimed to determine whether and how young and elderly muscle fibres differed in their response to denervation. I used a crush of the deep peroneal nerve to denervate and then measured the relative NCAM protein levels on the muscle surface after a two week survival period. 37 C57/B6j mice were used in total, 12 male and 25 female, split into two groups of 14 elderly (20 – 24 months) and 23 young adult (1 - 3 months) mice. Immunohistochemistry was used to detect both NCAM and its polysialylated variant, PSA-NCAM on the muscle surface. Fluorescent α-Bungarotoxin (α-BTX) was used to mark AChR at the NMJ, while rabbit anti-PSA-NCAM and rabbit anti-NCAM antibodies were used to detect the polysialylated and un-polysialylated forms respectively utilising AlexaFluor™ 488 and 568 secondary antibodies. Widefield fluorescence microscopy was used to visualise the tissues in cross-sections and whole mounts from four different muscle groups, and specimens were digitally photographed.

2.2 Ethics

All procedures and use of animals was approved by the animal ethics committee of the University of Otago.

2.3 Animals

The animals used in this project were male and female C57/B6j mice as well as a cohort of C57 mice carrying a genetically-encoded fluorescent reporter in a small subset of motoneurons (α-MN) (thy1-YFP, (Feng et al., 2000)) from the University of Otago breeding colony situated at the Hercus Taieri resource unit. The mice carrying the YFP label allowed a small subset of neurons to be visualised without prior staining, permitting visualisation of the crushed nerve during the surgical procedure. C57/B6j mice were chosen because of their extensive use in previous laboratory ageing studies. Prior work (Rowlatt et al., 1976) has shown that C57/B6j mice have an average lifespan of 26.7 months in both males and females (Ballak et al., 2014). In terms of lifespan equivalence, 24 months in mice is approximately 70 years in humans (Rowlatt et al., 1976). Research also shows that the age-related musculoskeletal changes observed in the C57/B6j strain correlate with similar changes in humans (Ballak et al., 2014). Male
and female mice were housed separately and all animals had free access to food, water and a simple environmental enrichment (plastic tube).

2.4 SURGICAL METHODS

2.4.1 Aseptic precautions

Aseptic techniques were used for all surgeries. All tools and equipment were autoclaved prior to use. Hibitane (Supplied by Hercus Taieri Resource Unit (HTRU), Dunedin, NZ) disinfectant was used on gloves, tools, coverings and anything leaving the aseptic field during surgery. During surgery, disposable gown, gloves, hat, mask and shoes were worn.

2.4.2 Anaesthesia

The animals were sedated with 4% isoflurane in a gas bell jar at a flowrate of 1 L per minute. Sedation was maintained using inhalation of isoflurane through a small nose cone. Deep anaesthesia was confirmed by loss of the toe-pinching reflex several minutes following sedation.

2.4.3 Surgical preparation

Once deep anaesthesia was confirmed, Tricin (Jurox Pty Ltd, Australia) antibacterial eye drops were applied to prevent drying and damage to the cornea (Figure 2.01A). Hair covering the surgical field was removed using an electric trimmer and hair removal cream (Marzena, NZ) to expose underlying skin (Figure 2.01B). The animal was stabilised on the surgical platform ensuring the face remained in the nose cone to maintain anaesthesia. The skin was cleaned and dried three times through the clockwise application with a cotton bud of Hibitane antibacterial disinfectant (Figure 2.01C). Finally, a plastic bag sterilised in Hibitane was placed over the entire animal to isolate the surgical field.

2.4.5 Nerve isolation

The sterile skin was resected from the anterior lower limb with microsurgery scissors, starting at the ankle and reflecting skin proximally up to the knee to expose the underlying tissues (Figure 2.01D). Connective tissue was bluntly dissected to reveal the
underlying tibialis anterior muscle, laterally located peroneal longus, lateral margin of the extensor digitorum longus (EDL) muscle and the surrounding perimysium. The remaining connective tissue between the EDL and the peroneal group was bluntly dissected to reveal the posteriorly located cavity housing the deep peroneal nerve and the anterior tibial artery and vein. A retractor was placed across the tibialis anterior and EDL muscle group to further reflect the muscles laterally. A retractor was also needed to reflect the peroneus group medially to increase the opening. This retraction exposed the bundle of nerves located just distal to the knee joint on the medial surface of EDL (Figure 2.01E). Within the bundle, the sensory branch of the deep peroneal nerve runs distally along the surface of EDL, while the motor branch bifurcates at the surface. One branch of the bifurcation continues proximally along the surface while the other enters the muscle at the point of bifurcation (Figure 2.01F).

2.4.6 Stimulation

The superficial branch of the bifurcation was isolated from the underlying connective tissue and a small bipolar stimulating electrode was placed under the proximal region of the nerve to allow for stimulation with a Grass SD9 stimulator (Grass Technologies, WI, USA). An evoked muscle response allowed for verification of the correct nerve and determined the voltage required to generate a twitch within the proximal region of EDL.

2.4.7 Nerve crush and double suture

2.4.7.1 Experimental crush

The proximal branch of the deep peroneal nerve was bluntly dissected away from the underlying connective tissue to allow placement of the micro-forceps under the nerve to enable a crush. The micro-forceps were marked with a vivid marker pen as a point of reference for the crush, and a standardised compression time of three seconds per crush was used for a consistent reproducible crush. A double suture of the crushed nerve was carried out to prevent axonal regrowth beyond the crush site. This process involved firmly tying 10 - 0 black monofilament surgical suture (AROSurgical, VT4A10Q07, CA, USA) proximally to the crush site with two surgical knots. A second suture was tied with two surgical knots around the crushed nerve area and closer to the muscle belly (Figure 2.01G, H). Upon completion of the double suture the stimulating electrode was replaced under the nerve and the predetermined voltage applied to
determine the efficacy of the denervation. If there was no visible twitch the crush was deemed effective. The retractors were then removed and the wound irrigated with sterile 0.9% NaCl saline solution (Ametech Ltd, NZ, A419). Following irrigation the wound was closed with a simple interrupted suture pattern using 4 - 0 braided suture (Johnson & Johnson©, NJ, USA) at regular intervals along the incision site.
Figure 2.01 Surgical procedure
Preparation of the surgical field involved stabilising the limb, hair removal and cleansing the skin (A-C). An incision was made and retractors were used to display the deep peroneal nerve as it enters EDL (D,E). The graphic also shows the bifurcation of the deep peroneal nerve, the location of the crush and proximal and distal double ligation (F-H).
2.4.7.2 Positive control crush

To measure the efficacy and effect of ligation alone the whole deep peroneal nerve innervating EDL was isolated and crushed in two animals (instead of just a small branch) using the previously described technique and double sutured using 10 - 0 monofilament (AROSurgical, VT4A10Q07, CA, USA). The same closing procedure was carried out to ensure aseptic technique was maintained before post-operative care was managed.

2.4.7.3 Sham procedure

The sham procedure was conducted on six animals and involved opening the lower limb and isolating the deep peroneal nerve following the same procedure performed in the experimental animals. The nerve was bluntly dissected away from the underlying connective tissue as if to allow for crush and double suture but was then left intact and the closing procedure carried out prior to post-operative care.

2.4.8 Post-operative care

All animals were given a bolus subcutaneous injection of Temgesic (Reckitt Benckiser®, Austria) (0.1 mL 0.1 mg/kg) (Buprenorphine - a semi-synthetic opioid agonist) as an analgesic for pain relief (Abrahamsson et al., 1983; Matsumiya et al., 2012) and a subcutaneous bolus of Amphoprim (supplied by HTRU, Dunedin, NZ) (0.1 mL 10.2 mg/kg) antibacterial to prevent infection prior to anaesthesia cessation. Finally, 0.1 mg/kg Lopaine (supplied by HTRU, Dunedin, NZ) (lignocaine) was topically applied to the skin with a cotton bud to further reduce pain and discomfort during recovery. Animals were then housed individually and monitored for 24 hours. The grimace pain scale was utilised to determine if further pain relief was required. The grimace pain scale is an objective scoring system of the stereotypical facial expressions of mice in response to pain (Matsumiya et al., 2012). It is based on changes in four distinctive areas: orbital tightening, nose/cheek flattening, ear changes and whisker change rated on a scale of pain severity (not present, moderate or obvious (Appendix, a6.03). A top-up of Temgesic (Reckitt Benckiser®, Austria) could be administered if the mice were seen to meet any of these criteria. Animals were then monitored by body weight, level of activity, presence of bleeding, the condition of wound and sutures daily throughout the two-week post-operative survival period. Based on these monitored parameters
additional doses of Amphoprim antibiotic (supplied by HTRU, Dunedin, NZ) could be administered (0.1 mL 10.2 mg/kg) at the first sign of infection and any further modifications to pain relief were adjusted with consultation with the HTRU vet.

2.5 TISSUE PREPARATION METHODS

2.5.1 Anaesthesia

The animals were anaesthetised via intraperitoneal injection of 0.3 mL of Pentobarb300 (supplied by HTRU, Dunedin, NZ) diluted to 30 mg/litre. Deep anaesthesia was then determined by loss of toe-pinch reflex several minutes later.

2.5.2 Transcardial perfusion

While the mice were deeply anaesthetised, the forelimbs, hindlimbs and tail were taped to a board to allow stable access to the torso and to prevent muscle shortening during fixation. Following this, an incision was made at the midline of the inferior abdomen. A cut was made moving superiorly towards the neck, ensuring the scissor tips were pointing up to cut only the skin and to prevent puncturing the abdominal wall. The skin was then peeled back to reveal the ribcage and cuts were made at the sternum moving superiorly towards each axilla (forming a “v” shape cut in ribcage). The ribcage was reflected and held in place with a haemostat to reveal the mediastinum. An incision was made in the right atrium to allow the flow of venous blood from the body. The heart was held stable and a 23 gauge needle was inserted into the tip of the left ventricle. Heparinised phosphate buffered saline (PBS) with 20 mg sodium nitrate was preheated to body temperature (37°C) to prevent vasoconstriction (Appendix, a6.04) and infused for one minute at a rate of 6 ml/min using a peristaltic pump to prevent clotting in small vessels. Sodium nitrate acts as a nitrate donor and promotes vasodilation of the small vessels and capillaries through activation of eNOS (epithelial nitric oxide synthase) to ensure thorough fixation. The perfusion was then switched to preheated (37°C) fixative (1% paraformaldehyde in 0.1 M phosphate buffer) for five minutes during which a step wise increase in flow rate from 6 mL/min to 10 mL/min allowed rapid and complete fixation. Following fixation the mice were skinned, eviscerated and placed in a tube containing 1% fixative in PBS, before being placed in the fridge (at 4°C) overnight (Appendix, a6.07).
2.5.3 Muscle excision

Four muscles were selected for excision from the perfused mice: soleus and extensor digitorum longus (EDL) from the hind limb, and sternomastoid and cleidomastoid from the neck. Following extraction the muscles were placed in the fridge (at 4°C) in a tube containing 10% sucrose in PBS as a cryoprotectant to minimise ice crystal formation during the freezing process.

2.5.3.1 Soleus

The soleus is believed to be a slow postural muscle in the hind limb of many animals including the mouse and is a muscle that typically undergoes natural degeneration with age (Ariano et al., 1973). Dissection required incision at the lateral aspect of the ankle, followed by blunt dissection of the tibialis anterior from the gastrocnemius. The achilles tendon of the triceps brachii was cut and the gastrocnemius was reflected posteriorly to reveal the soleus sitting on the medial surface. The superior tendon could then be located and cut before the soleus was bluntly dissected from the gastrocnemius as it was pulled towards the distal tendon. It could then be excised by severing the distal tendon at its attachment point (Appendix, 6.2).

2.5.3.2 Extensor Digitorum Longus (EDL)

The EDL muscle comprises a higher proportion of type II fast fibres than the soleus muscle and is involved in extension of the toes, with its insertion at the distal ends of the phalanges (Cederna et al., 2001). Excision required identification of the lateral margin of the tibialis anterior so that it could be bluntly separated moving superiorly from the edge of gastrocnemius to the knee. The edge of tibialis anterior (TA) was then reflected to reveal EDL on the medial surface. The inferior tendon could then be cut and EDL was pulled towards its superior tendon insertion at the tibia and bluntly dissected from the connective tissue attaching it to TA. The superior tendon could then be severed to allow for excision at the head of the tibia (Appendix, 6.2).

2.5.3.3 Sternomastoid and Cleidomastoid

These anterior muscles of the neck allow for stabilisation and rotation of the head and are less susceptible to age-related degeneration than limb muscles (Sheard & Anderson,
2012). Excision requires the superficial adipose tissue and thyroid gland to be removed to reveal the sternocleidomastoid group. The forceps were placed between the two overlapping muscles and used to separate the two muscles by bluntly separating any connective tissue. Sternomastoid lies anterior to cleidomastoid and requires an incision to be made at its distal insertion at the clavicle/sternum. It can then be bluntly dissected back towards its proximal insertion below the ear on the mastoid bone. The cleidomastoid muscle consists of two heads that insert into the clavicle. These are cut as close as possible to the clavicle before being bluntly dissected back to their proximal insertion with the sternomastoid group at the mastoid bone below the ear (Appendix, 6.2).

### 2.5.4 Freezing and sectioning procedure

The muscles were placed in 10% sucrose in PBS overnight to provide cryoprotection. Freezing boats were made from aluminium foil filled with Optimal Cutting Temperature (O.C.T) cryomatrix (Sakura®, Tissue-Tek, Netherlands) compound which acts as the embedding medium for the tissues. Tissues were cleaned, trimmed and oriented longitudinally to expose the motor endplate band of the muscle and were placed in the aluminium boats. L-Isopentane was cooled to approximately -170°C by liquid nitrogen and was used to snap freeze the muscles embedded in pre-chilled (4°C) cryomatrix though partial immersion of the aluminium boats. Partial immersion prevented the cryomatrix from cracking as it froze, as it allowed the surface of the block to be last to freeze and thereby allowing for extrusion of unfrozen cryomatrix as surface layers contracted during freezing. The frozen tissue blocks were stored in a -80°C freezer until required for sectioning. Sectioning of the tissue blocks was carried out using a cryostat (Lecia CM1850) at -20°C. The ribboned sections were cut in either transverse or longitudinal planes at 10 μm and adhered to Vectorbond™ (Vector laboratories Inc, CA, USA) treated microscope slides. To confirm sections were within the endplate region of the muscle, cholinesterase stain (Appendix, a6.03) was used to identify the post-synaptic apparatus. Once within the endplate region the transverse sections were cut using a parallel sectioning approach to provide comparable tissue samples for identification of polysialylated neural cell adhesion molecule (PSA-NCAM), un-polysialylated neural cell adhesion molecule (unNCAM), synaptophysin (SYN) and voltage-gated sodium channel 1.5 (NaV1.5) (see Figure 2.04). Parallel sectioning allowed
observation of fluorescence present for all four proteins at or near a single NMJ, allowing a more accurate assessment of any changes in protein expression. Sections were then left to dry on to slides for 20 min before rehydration in TBS (Appendix, a6.13/14).

2.5.5 Denervation

To determine the efficacy of the surgical crush, a series of techniques were utilised to trace the path of the deep peroneal nerve as it entered the EDL muscle of two animals.

2.5.5.1 Lipophilic dye, DiO

A multicolour neurotracer tissue labelling kit of lipophilic dyes (DiO) (Life Technologies, N22884, CA, USA) was used to trace the path of the nerve as it entered the muscle. The lipophilic dye DiO was used as an anterograde neuronal tracer as it has been shown to progress in an anterograde fashion along neurons at 0.2 - 0.6 mm per day (Balice-Gordon et al., 1993). This occurs since the fluorescent carbocyanine dye is attached to two alkyl groups that determine the affinity to both water and the lipid of the cell membrane. The DiO structure makes it immiscible with water allowing it to move within the membrane via diffusion (Honig & Hume, 1989). DiO fluoresces when excited by blue light (470 nm) and emits green light (500 - 550 nm) to allow visualisation under a fluorescence microscope (Figure 2.02).

![Figure 2.02 DiO excitation emission spectrum](image)

The fluorescent neurotracer DiO is excited at 470 nm and emits light at 500-550 nm.
Due to DiO insolubility in water, it was dissolved in tetruglycol as per Venters et al. (2013) (Sigma, 105H0135) before the paste was applied with forceps into the dried and exposed severed proximal ending of the deep peroneal nerve where it entered extensor digitorum longus.

### 2.5.5.2 Clearing Protocol

A clearing protocol was also employed to provide verification of successful nerve crush. It utilised a series of different procedures including BABB, Scale and SeeDB to clear the muscle tissue revealing the underlying nerves stained using antibody to neurofilament 160 kDa (abcam®, ab64300, Cambridge, UK).

The clearing protocols used Benzyl Alcohol Benzyl Benzoate (BABB)/Murray’s clear, Scale or SeeDB to reduce the light scattering properties of muscle. Muscle is densely packed with substances that have a high refractive index e.g. collagen and elastic fibres, while the surrounding compartments including the cytoplasm have a low refractive index due to their high water content (1.333), thereby causing light scatter and tissue opacity (Zhu et al., 2013). Clearing protocols add a high refractive index solution (BABB, Urea and fructose) to bring the refractive index of the whole tissue to an equilibrium, thereby allowing light to pass relatively unscattered through the tissue allowing it to appear more transparent (Zhu et al., 2013).

#### 2.5.5.2.1 BABB protocol

Excised muscles underwent three five-minute 1x Tris Buffered Saline (TBS) washes before overnight (OVN) incubation in anti-neurofilament 160 kDa primary antibody. Tissues then went through a wash cycle that included three five-minute 1x TBS washes followed by five one-hour 1x TBS washes. Tissues were then incubated in secondary antibody Alexa 488 (Life Technologies, California, USA, no. A-11070) overnight (OVN). A second identical wash protocol was carried out prior to stepwise dehydration using methanol, which comprised one five-minute wash at 50% and three 30-minute washes at 100%. Following dehydration tissues were left to incubate in a 2:1 mix of benzyl benzoate and benzyl alcohol until cleared (Appendix, a6.01) (Kardon, 1998). In addition to the protocol used by Kardon (1998), a pre-primary antibody incubation in sodium borohydride (SBH) was used for quenching auto-fluorescence and involved a seven-minute incubation in SBH at 1% prior to primary antibody incubation.
The addition of a dehydration step within the BABB protocol is crucial as evidence suggests that it further equalises the refractive indices by drawing water away from the interstitial spaces (Klymkowsky & Hanken, 1991; Zhu et al., 2013).

2.5.5.2.2 Scale protocol

Scale is a urea-based clearing agent thought to be superior to BABB as antibody fluorophores are stable in urea (Ke et al., 2013). The protocol for scale is similar to BABB with primary and secondary antibody incubations followed by incubation until clear in Scale solution. The Scale A-2 solution was used, composed of 4 M Urea, 10% (wt/vol) glycerol and 0.1%(wt/vol) Triton X-100 (Appendix, a6.09) (Hama et al., 2011). Tissues were incubated for >2 weeks at 30°C in the dark.

2.5.5.2.3 See Deep Brain protocol

See Deep Brain (SeeDB) is a water-based clearing agent that utilises increases in fructose concentrations until saturation (Figure 2.03). Each stepwise increase involved an eight-hour incubation from 20 - 100% before a 24-hour incubation in SeeDB solution (Appendix, a6.12). Fructose has a refractive index of 1.490 at room temperature and is highly soluble in water, which allows it to align the refractive index of water (1.333) closer to the refractive index of the proteins within the extracellular compartments without quenching the fluorophores (Ke et al., 2013; Ke & Imai, 2014).
2.5.5.3 Parallel section immunohistochemistry

The efficacy of denervation was determined using a series of primary antibodies run on parallel muscle sections to allow identification of multiple proteins at a single endplate region (Figure 2.04). Synaptophysin (SYN) and NaV1.5 were stained to mark the presynaptic terminals and voltage-gated sodium channels respectively, the loss of SYN at a NMJ with the retention of a post-synaptic apparatus as marked by α-BTX would indicate absence of the neural input from the muscle fibre, and therefore effective denervation. While NaV1.5 has previously been shown to be a marker for denervated muscle fibres (Rowan et al., 2012), co-localisation of NaV1.5 around muscle fibres lacking SYN staining would provide further evidence of successful denervation. A final slide of negative control sections were cut and run with no primary just secondary antibody to ensure the specificity of secondary antibody binding.
2.5.6 Immunohistochemistry

The immunohistochemical (IHC) optimisation protocol included determining the concentration, temperature, time and requirement for epitope retrieval of each antibody. The optimal conditions were determined via tissue array on 1, 2 and 4% paraformaldehyde fixed tissues. All antibodies were diluted in TBS as TBS neutralises tissue auto-fluorescence and creates cleaner immunohistochemistry than PBS.
2.5.6.1 Un-polysialylated NCAM (unNCAM) (Proteintech™ no. 14255-1-AP)

UnNCAM is the unmodified form of NCAM expressed as a 120 or 140 kDa isoform in muscle. Its functional properties involve homophillic binding to other NCAM molecules at the neuron, Schwann cells or satellite cells, or heterophillic binding to proteins of the extracellular matrix (Covault et al., 1986; Walsh & Doherty, 1991).

The unNCAM antibody (Proteintech™, IL, USA, no. 14255-1-AP) optimisation protocol required four permutations including a standard run of three-hour incubations of primary antibody at 1 in 250, 400, 500 and 1000 dilution and a four-hour or OVN secondary antibody incubation of anti-species conjugated Alexa 488 (Life Technologies, N22884, CA, USA) at 1 in 250, 500 and 1000 at room temperature or 4°C respectively. The second permutation used Heat Induced Epitope Retrieval (HIER) in both Tris- and citrate-buffered solutions (Appendix, a6.08). Antigen retrieval is used for formaldehyde-fixed tissues as formaldehyde fixation may mask the epitope or binding region of the antigens by altering the secondary folding of the protein thereby affecting the efficacy of the antibody binding (O’Leary et al., 2009). The rationale for HIER is that heating the solution can denature the cross-linked proteins and therefore unmask the epitope binding region on the antigen for antibody binding (O’Leary et al., 2009). Tris HIER involved a 20-minute incubation in Tris buffer at 90°C followed by a cooling stage to 30°C for four hours. The aforementioned immunohistochemical procedure was then conducted (Appendix, a6.13).

The third permutation involved a permeabilisation step with a permeabilization solution (Appendix, a6.05) which aimed to improve diffusion into the tissue sections. The final permutation involved four-minute incubations in 0.5% sodium dodecyl sulfate (SDS) as a further epitope retrieval step. SDS denatures the sample through the addition of negative charges to the amino acids within the lipid bilayer, which frees the antigen and exposes the epitope binding region to the antibody (Robinson & Vandré, 2001; Caprette, 2012). The optimal concentration and duration for pre-incubation was determined by multiple tissue array with a series of different concentrations of SDS at 0.5 and 1% at four and five min. Tissue arrays were also used to determine the efficacy of pre or post incubation with 0.5 mg/mL of SBH for seven minutes. SBH reduces aldehydes and ketones to quench auto-fluorescence in cryostat sectioned tissues (Clancy & Cauller, 1998; Spitzer et al., 2011) and was therefore investigated to improve
visualisation of NCAM antibodies. The optimum protocol was conducted at room temperature and involved a 4-minute pre-incubation of 0.5% SDS, a four hour primary incubation at 1:400 and finally a four-hour secondary antibody and fluorescent α-BTX incubation at 1:500 (Invitrogen, Life Technologies, CA, USA).

2.5.6.2 Polysialylated NCAM (PSA-NCAM) (Millipore™, MA, USA no. AB5032)

PSA-NCAM has undergone a post-translational addition of a long chain sugar, increasing its molecular weight from 120 to 250 kDa. The modification acts to sterically hinder NCAM homophilic and heterophilic binding properties and instead favour the intracellular signalling properties, hence it is referred to as “active NCAM”.

Optimisation of anti-PSA-NCAM antibody (Millipore™, MA, USA no. AB5032) binding also involved dilution and antigen retrieval permutations. Dilutions consisted of a three-hour primary antibody incubation at 1 in 250, 500 and 1000 followed by either a four-hour or OVN secondary antibody incubation with antispecies conjugated Alexa 488 at 1 in 250, 500 or 1000 at room temperature and 4°C respectively. A blocking step utilising both bovine serum albumin (BSA) and SDS was used to optimise the immunofluorescence. BSA blocking involved an overnight incubation before primary incubation to inhibit any non-specific antibody binding. The SDS concentration was determined following the aforementioned permutations used for unNCAM optimisation. The optimum protocol followed the same protocol used for unNCAM immunofluorescence.

2.5.6.3 Anti-NaV₁.5 (NaV₁.5) (Biossusa bs-9445R-A488 and Alomone labs ASC-005)

Anti- NaV₁.5 antibody was used to detect the tetrodotoxin-insensitive voltage-gated sodium channel NaV₁.5/SCN5A/SkM2 which is highly expressed within cardiomyocytes. This channel plays an essential role alongside gap junction proteins in action potential generation and conduction between cardiomyocytes (Figure 2.05) (Petitprez et al., 2011; Rhett et al., 2012). NaV₁.5 expression in skeletal muscle is characterised by high mRNA levels during development, low level expression in normal adult muscle and shows a transient period of up-regulation in response to denervation (Kallen et al., 1990; Yang et al., 1991; Rowan et al., 2012). NaV₁.5 staining is used as another marker of muscle fibre denervation.
Optimisation of the Biossusa NaV\textsubscript{1.5} antibody (Biossusa, MA, USA, bs-9445R-A488) required a series of permutations tested on both heart (as a positive control (Yang \textit{et al.}, 1991)) and elderly skeletal muscle (Rowan \textit{et al.}, 2012). Variations in concentration included 1 in 50, 100, 200, 500 and 1000 for time periods varying from 3 to 12 hours at room temperature or up to 24-hours at 4°C. The effect of tissue fixation on antibody efficacy was tested on tissue at 0, 1, 2 and 4% paraformaldehyde and with Bouins solution. The use of Bouins was employed as data have suggested that immunohistochemical staining is influenced by the type of fixative and the storage post-fixation (Atkins \textit{et al.}, 2004). At the antibody concentration of 1 in 100 a series of different antigen retrieval methods were tested prior to incubation, including HIER with Tris buffer and citrate buffer solutions, SDS at 1 and 0.5% were also trialled. Following Biossusa advice, a permeabilization step with Tris and Triton X-100 (Appendix, a6.05) for 30-minutes prior to incubation in primary antibody was investigated. Finally, heart and elderly skeletal muscle specimens were paraffin embedded and cut at 4 μm thick sections. The slides were taken through a series of permutations including varying concentrations 1 in 50, 100 and 200, epitope retrieval steps using both HIER with citrate buffer, and SDS antigen retrieval.

A second NaV\textsubscript{1.5} antibody from Alomone Labs (Alomone Labs, Jerusalem, Israel, ASC-005) was tested and optimised. Use of the Alomone NaV\textsubscript{1.5} antibody followed optimisation protocols involving three-hour primary antibody incubations with varying concentrations and temperature. Permutations included 1 in 100 and 200 incubations at room temperature and 4°C respectively, followed by a four-hour secondary antibody incubation of either anti-species conjugated Alexa 488 or Alexa 568 at room temperature. The optimal protocol was identified to be 1 in 500 primary antibody rabbit anti-NaV\textsubscript{1.5} incubation for four-hours at room temperature, followed by four hour incubation in Alexa 488 goat anti-rabbit secondary antibody also at room temperature (Figure 2.05).
2.5.6.4 Synaptophysin (SYN) (Abcam no. ab32594)

Synaptophysin is a synaptic vesicle protein and is widely used as a pre-synaptic nerve terminal marker allowing identification of the neural input to each muscle fibre. Optimisation of the SYN antibody involved concentration permutations including three-hour primary antibody incubation at 1 in 200, 500 and 1000 followed by OVN secondary antibody incubation at 1 in 500 and 1000 at 4°C. The optimum protocol was a three-hour primary incubation at 1 in 500 at room temperature followed by a four-hour incubation with secondary antibody Alexa 488 and fluorescent α-BTX both at 1 in 500 at room temperature.
2.5.6.5 Wheat Germ agglutinin (WGA) (Life technologies ref.W11263)

Wheat germ agglutinin is a lectin (monosaccharide) (in our case conjugated to an Alexa Fluor 350 fluorophore) that binds sialic acid residues located on glycoproteins. Sialic acid as previously mentioned is added via post-translational modification to series of structures including PSA-NCAM and to the plasma membrane of muscle fibres. When the conjugated Alexa Fluor 350 is excited with 350 nm light (UV) it causes 450 nm emission and visualisation of blue staining. A 1.0 mg/mL preparation of WGA was prepared via dissolving 5.0 mg of WGA in 5.0 mL of 1x PBS. WGA can then be added to sections and viewed within moments to visualise muscle fibres (Figure 2.06)

2.5.7 Microscopy and photography

Slides were viewed on an upright compound epifluorescence microscope (Olympus BX-50, Olympus® Corporationm Tokyo, Japan) equipped with a CoolLED fluorescence illuminator (pE-1 excitation system, CoolLED Ltd, NY, USA) and digitally photographed with a SPOT-RT™ Slider (Diagnostic Instruments Inc., MI, USA) scientific microscope camera. The slides were excited at each of three wave lengths 535, 470 and 365 nm, and their resulting emissions photographed with three dichroic cube filters within the microscope, including the Wide Band Interference (WBI) blue (excitation range 515 - 700 nm), WBI green (excitation range 580 - 700 nm), and narrow band interference UV (excitation range 360-370 nm) filters. The images were viewed and imaged using the x 10 dry and x 20, x 40 and x 60 oil immersion lenses. The optimal illumination and photography parameters for the entire run was determined based using the brightest sample for each antibody (for example, LED brightness, magnification and exposure time). These parameters once determined were kept constant across all imaging days and specimens to allow comparison of data collected. Any photomerging of cross-sections of muscle fibres involved taking multiple images that were then stitched together and merged using Photoshop® (CS4) (Adobe® systems Inc., CA USA).
2.5.8. Image analysis and statistics

2.5.8.1 Fibre breadth analysis

Image analysis of the fibre breadth was completed using Photoshop® add-on, FoveaPro 4.0 (Reindeer Graphics, NC, USA). The aim was to establish the breadth of the fibres stained using WGA in order to associate a change in fibre size with the changing innervation status. In order to do this following imaging of PSA-NCAM, unNCAM, SYN and NaV1.5 the slides were de-coverslipped by placement in 1x TBS for 10 minutes before being re-coverslipped with WGA to stain the lectins within the plasma membrane, resulting in the muscle fibre membranes fluorescing blue (Figure 2.06). Using a thresholding filter, Photoshop® allowed us to obtain a binary image of the muscle fibres where the fibres are represented as black blobs on a white background. Following this using FoveaPro’s “measure all features” routine we were able to obtain the breadth of each of the fibres. Breadth was used rather than area or perimeter as it gives a consistent representation of the fibre diameter and avoids the potential confounder of fibre orientation within the section, shown in a theoretical spaghetti model below (Figure 2.07).
Figure 2.07 Breadth justification

Figure A) highlights the differences between the actual area, perimeter and breadth values and the observed values when measured using Photoshop© Fovea Pro 4.0 add-on. Each point on the graph (1,2,3...) corresponds to a point on the 3D model of a curved cylinder (B) to show how the changes in shape based on the point of section affects the measurements taken.
2.5.8.2 **Fibre breadth statistics**

To extrapolate measureable data from the binary images the fibre breadths within the denervation innervation cohort and between young and elderly mice were plotted on a histogram. Histograms allow both qualitative and quantitative identification of variations in the data sets distribution based on the size of the bin widths. To determine the optimal bin width for the histogram, we used the adapted form of Sturges’ formula that utilises the number of items (N) and the range of those items (R) to determine the optimal interval (k) (Sturges, 1926).

\[ k = \frac{R}{1 + 3.322 \log N} \]

This has since been modified by Scott, 1979 to account for the standard deviation (\( \sigma \)) of the number set (n). Allowing for an extrapolation of the optimal bin width while maintaining sensitivity within the defined sample size.

\[ k = \frac{3.5\sigma}{n^{1/3}} \]

However, as with each animal (sample) there are variations in both the sample size and standard deviation. Therefore, the bin width used was established by averaging a sample from each group: denervated EDL young/elderly and innervated young/elderly.
2.5.8.3 Immunohistochemical analysis

Analysis of the immunofluorescence staining involved looking at a series of different aspects of the staining produced by each antibody in response to both surgical denervation and sarcopenia.

2.5.8.3.1 PSA-NCAM analysis

The analysis of PSA-NCAM involved looking at both junctional and cytoplasmic expression levels of PSA-NCAM. Junctional measurements were taken at the NMJ demarcated by the presence of bungarotoxin. The region of interest (ROI) was demarcated using the free form pen tool in Image J (National Institute of health, MD, USA), and brightness levels within the ROI were then measured. The area within the selected area was measured for mean grey value (0-255), standard deviation (SD) and area (Figure 2.08).

The other measurement required for NCAM analysis was the staining level (brightness) within the muscle fibre, to account for the amount of cleaved NCAM present within the muscle. This was measured via selecting the entire muscle fibre area excluding the previously measured junctional region again producing a mean grey value (0-255), SD and an area (Figure 2.09).
2.5.8.3.2 UnNCAM analysis

Analysis of unNCAM followed a similar procedure to PSA-NCAM. Junctional levels of unNCAM were measured to determine the relative levels present between the young and elderly denervated mice (Figure 2.08). Unlike PSA-NCAM, unNCAM however is not cleaved and only acts as a cell-adhesion molecule therefore measurements of the extra-junctional unNCAM levels were measured instead, as increases in extra-junctional unNCAM levels post-denervation have been seen in young animals (Covault & Sanes, 1985). This involved using the segmented line tool in Image J with a line thickness of five pixels. The line could then be traced around the extra-junctional regions either side of the junctional region. At which point the averaged grey value and area within the five pixel line were measured (Figure 2.10).

Determination of the average grey level, SD and area from the three regions (junctional; extra-junctional; and cytoplasmic) allows for comparison of the relative amount of protein present as indicated by brightness of immunofluorescence between surgically denervated young and elderly mice. Combining the grey scale and area as a ratio given as the protein per pixel (PPP) also allows an indication of the concentration of protein within the defined regions.
2.5.8.3.3 NaV₁.₅ analysis

NaV₁.₅ immunofluorescence was measured as either presence or absence at muscle fibres previously deemed to be denervated. Denervation was determined via loss of the pre-synaptic terminal at each NMJ indicated by absence of the SYN immunofluorescence (Figure 2.11).
2.5.8.4 Statistical Analysis

The statistical analysis was conducted using GraphPad Prism™ 6 (GraphPad Software Inc., CA, USA) to determine the presence of a normal distribution amongst the collected data sets (normality) using a D’Agostino & Pearson omnibus normality test. This test allowed me to determine whether a standard parametric one/two way analysis of variance (ANOVA) was sufficient to determine significance between data sets. Once a normality test was completed, and a non-normal distribution was determined the raw values were transformed by Prism into log values to remove the effects of potential outliers and generate a normal distribution. If a normal distribution could not be established across the data sets a non-parametric analysis such as a Kruskal-Wallis one-way ANOVA and a post-hoc Dunn’s multiple comparison analysis were used to determine significance assuming the sample sizes were sufficient. For the elderly PSA-NCAM analysis, as only two variables could be collected (denervated fibres from surgical EDL and innervated fibres from control EDL) a Kruskal-Wallis could be used to conduct statistical comparisons. Following normality tests a non-parametric Mann-Whitney U test was used to compare the variables. The statistical tests allowed determination of any significant differences between ages and the effects of denervation as a derivative of the corresponding staining intensity.

GraphPad prism was also used to analyse the fibre counting data sets. The use of a normal t test was not adequate as a result of the large sample size generated. As all of the muscle fibres were counted within the entire muscle, the average from each muscle was used as representation of the animal. From the 12 animals a non-parametric analysis such as a Kruskal-Wallis one-way ANOVA and a post-hoc Dunn's multiple comparison analysis were used to determine the presence of any significant differences between the two ages and the effects of denervation on fibres as a derivative of the corresponding fibre breadth distributions. Differences from all analyses were considered statistically significant if the tests returned p values of less than 0.05.
3. CHAPTER THREE

RESULTS
3.1 Immunohistochemistry

The results presented below were collected from fluorescent immunostained sections of frozen skeletal muscle. Before results could be collected, the protocols and reagents underwent optimisation to determine the optimal concentration, incubation time and temperature, and the requirement for antigen retrieval. The outcomes of the different optimisation permutations are described below.

3.1.1 Un-polysialylated-Neural Cell Adhesion Molecule optimisation

The un-polysialylated-Neural Cell Adhesion Molecule (unNCAM) optimisation protocol discussed in methods 2.5.6.1 included different concentrations of Sodium dodecyl sulphate (SDS) and sodium borohydrate (SBH). I found that a protocol involving a four-minute pre-incubation in SDS, before a four-hour primary and four-hour secondary antibody incubation at room temperature using 1 in 400 and 1 in 500 concentrations respectively, yielded the best histology and signal-to-noise ratio (Figure 3.01A).

![Figure 3.01 UnNCAM optimisation](image)

All tissue sections were run with unNCAM primary at 1 in 400 and secondary Alexa 488 at 1 in 500 concentration. A) UnNCAM sections were pre-incubated with 0.5% SDS for 4 minutes revealing clean staining. B) Bungarotoxin (α-BTX) staining demarcates the post-synaptic apparatus of the NMJ. C) UnNCAM and α-BTX merged imaged showing co-localisation of the pre and post-synaptic apparatuses. D) Minimal staining is present in a standard run of 1 in 500 primary (white arrow). E) UnNCAM with pre-incubation of 1% SDS staining show a large number of junctions present, however staining was not as optimal as 0.5% SDS (white arrow). F) UnNCAM with pre-incubation of SBH for 7 minutes shows a poor signal to noise ratio of the immunohistochemical staining.
3.1.2 Polysialylated-Neural Cell Adhesion Molecule optimisation

The polysialylated-Neural Cell Adhesion Molecule (PSA-NCAM) antibody optimisation protocol required pre-incubation with SDS for the most effective staining. Staining of the permutations discussed in methods 2.5.6.2 is depicted below (Figure 3.02). The different permutations investigated resulted in a varying appearance of staining. The staining pattern evident without pre-incubation was faint and featured non-specific

![Figure 3.02 PSA-NCAM optimisation](image)

All tissue sections were run with NCAM primary antibody and secondary Alexa 488 at 1 in 500 A) NCAM immunohistochemistry with 0.5% pre-incubation of SDS for 4 min. B) α-BTX marks the post-synaptic apparatus. C) Overlaid image of NCAM and α-BTX show pre and post-synaptic co-localisation. The following are images of ineffective permutations investigated, D) NCAM staining without pre-incubation. E) NCAM following heat-induced antigen retrieval F) NCAM with SDS pre-incubation at 1% for 4 min. G) NCAM with SDS pre-incubation at 1% for 5 min. H) SDS pre-incubation at 0.5% for 5 mins. I) NCAM with SBH 7-minute pre-incubation, J) 7-minute pre-incubation in SBH followed by 5 min incubation in 1% SDS. K) 5-minute incubation in 1% SDS followed by 7-minute incubation in SBH. Scale bar 50 μm.
staining (Figure 3.02D). Implementation of a HIER step removed all NCAM staining from the tissue (Figure 3.02E), while pre-incubations with 1% SDS for 4 and 5 min resulted in poor signal-to-noise ratios in imaged sections (Figure 3.02F & G respectively). Investigation of using both SDS and SBH resulted in effective staining but altered the fluorescence of the tissues making the signal-to-noise ratio poor, regardless of the order (Figure 3.02 I, J, K & L). The optimal protocol involved a four-minute pre-incubation in 0.5% SDS followed by a four-hour primary and four-hour secondary incubation, at 1 in 500 at room temperature that gave clean and concise immunohistochemistry that co-localised cleanly with bungarotoxin (Figure 3.02A, B & C) as five-minute incubations with 0.5% SDS resulted in less clear staining.

3.1.3 Synaptophysin optimisation

The optimisation of the SYN antibody required several different concentrations including 1 in 250, 500 and 1000 (Figure 3.03A, B & C). An optimal concentration of 1 in 500 with 1 in 500 secondary Alexa 488 was determined (Figure 3.03B).

![Synaptophysin optimisation](image)

Figure 3.03 Synaptophysin optimisation
Concentration permutations for SYN antibody with a 1 in 500 concentration of secondary antibody Alexa 488. A) 1 in 250 concentration had faint staining, B) 1 in 500 concentration showed clear and distinctive SYN staining, C) 1 in 1000 concentration produced no change in fluorescence from 1 in 500. All sections were incubated for 4 hours at room temperature. Scale 50 μm.

3.1.4 NaV1.5 optimisation

Rowan et al. (2012) previously presented un-convincing immunolabelling of NaV1.5 co-labelled with MHC-1 in denervated rat muscle due to the apparent strong bleed through from the red channel (Figure 3.04F). Therefore, one of my aims was to determine the efficacy of the NaV1.5 antibody as a marker for denervation. Two antibodies were investigated to optimise NaV1.5 immunohistochemistry (IHC). The BiossUSA (bs-9445R-A488) antibody employed normal fixation, SDS antigen retrieval, Heat Induced Epitope
Results

Retrieval (HIER) (Figure 3.04A) and bouins fixative (Figure 3.04B), on both skeletal muscle (Figure 3.04A) and heart tissue to no avail (Figure 3.04B). The Alomone (ASC-005) antibody was tested on heart tissue as a positive control and showed distinctive cardiomyocyte outlines at a 1 in 500 concentration (Figure 3.04C). Therefore I proceeded with this antibody. After incubation of surgically denervated extensor digitorum longus (DEDL) tissue, a subset of fibres labelled in a manner consistent with denervation and showing marked improvement over images depicted in Rowan et al. (2012) (Figure 3.04D, E).
NaV\textsubscript{1.5} at 1 in 500 from their respective companies produced various extent of staining. A) BiossUSA NaV\textsubscript{1.5} with HIER pre-incubation on elderly skeletal muscle (scale 50 μm for image A-D) B) BiossUSA NaV\textsubscript{1.5} on bouins fixed heart tissue C) Alomone NaV\textsubscript{1.5} on paraformaldehyde fixed heart D) Alomone NaV\textsubscript{1.5} in young DEDL skeletal muscle E) 40x magnification of image D showing encircled fibres (solid arrows) F) Image taken from Rowan et al. (2012) with NaV\textsubscript{1.5} in green (solid arrow) and MHC I fibres in red with the lack of NaV\textsubscript{1.5} staining (hollow arrow). Scale bars 50 μm for images E and F.
3.2 Denervation

Examination of NCAM in young and elderly tissues required validation of the surgical crush procedure and a series of histological and IHC validation procedures were tested; the efficacy of each one is described below.

3.2.1 Lipophilic dye (DiO) nerve tracing

DiO is a lipophilic fluorescent substance which is widely used as an anterograde nerve tracer in fixed preparations (Honig & Hume, 1989; Gan et al., 2000). In my hands, DiO did not diffuse far enough along the axons of interest to be useful even when allowing a 12-week incubation period. The poor labelling and long incubation time combined for a low quality result (Figure 3.05).

Figure 3.05 DiO staining of the Deep peroneal nerve
A DiO stained path of the deep peroneal nerve (white arrows) as it travels within the muscle following entry from the surface (red arrow).
3.2.2 Clearing results

As the DiO neurotracer was ineffective at distinguishing the path of the deep peroneal nerve, I instead pursued a wholemount immunohistochemical approach coupled with large-volume tissue clearing protocols.

3.2.2.1 Benzyl Alcohol Benzyl Benzoate

The BABB clearing technique (Kardon, 1998) (Methods 2.5.2.1) produced nearly transparent muscles. However, there was high autofluorescence and the anti-neurofilament IHC (Abcam, ab64300) signal was obscured in deep layers (Figure 3.06).

3.2.2.2 Scale

Scale was tested as an alternate large-volume tissue clearing agent. Published work indicates that long-term incubations (up to 3 months) are required to clear tissues (Hama et al., 2011). I found this to be true as the muscle tissue did not clear sufficiently within the time available and resulted in poor transparency (Figure 3.07A).

3.2.2.3 See Deep Brain (SeeDB)

Finally, I investigated the See Deep Brain (SeeDB) clearing protocol. SeeDB, like BABB, resulted in tissues that appeared transparent. However, the specimen was highly autofluorescent under widefield fluorescence microscopy (Figure 3.07B).
3.2.3 Sectioning

As the clearing procedures were ineffective, I decided to cut parallel sections of DEDL and contralateral innervated extensor digitorum longus (EDL I) of the same mouse for parallel processing with NaV$_{1.5}$ antibody. These sections tested NaV$_{1.5}$ as a marker of denervation verified by the pre-synaptic terminal marker SYN to validate innervation status. The NaV$_{1.5}$ antibody produced distinctive specific staining around the perimeter of a subset of fibres in DEDL tissue (Figure 3.08A) while no staining was present in the EDL I muscle (Figure 3.08B). This confirmed that NaV$_{1.5}$ was specific to denervated fibres. Another supporting observation was the significant reduction in fibre diameter between DEDL (Figure 3.08C) and EDL I muscle tissue. Upon further investigation, NaV$_{1.5}$ encircled fibres were found to have intact post-synaptic apparatuses (Figure 3.08G) but were missing their corresponding pre-synaptic terminal (Figure 3.08D) which indicates recent denervation.

![Figure 3.07 Scale and SeeDB clearing](image)

A) Scale was unable to produce clarity in the tissue as the fibres (hollow arrow) were still visible in the whole mount preparations of sternomastoid. B) SeeDB produced a visibly clear muscle which autofluoresced under the microscope. Partially visible neurofilament staining of the nerves (solid arrow) is seen within the muscle belly.
Figure 3.08 Denervation status

A) NaV_{1.5} antibody fluorescent staining (green) encircled the denervated fibres within the DEDL muscle. Scale bar 50 μm.
B) NaV_{1.5} immunohistochemical staining is absent in the EDL muscle. C) NCAM staining of DEDL muscle shows a significant reduction in muscle fibre diameter in DEDL muscle. D) NCAM staining from EDL I muscle shows large diameter fibres (scale 50 μm). E) The absence of SYN staining in DEDL at a NMJ demarcated by both the Bungarotoxin (red, F) and wheat germ agglutinin (blue, G) staining of the post-synaptic apparatus in the DEDL muscle. Scale bar 25 μm.
3.3 Fibre size distribution

Since muscle fibre atrophy is one sign of denervation (Rowan et al., 2012), it was helpful to compare fibre size distribution between DEDL and EDL muscles of both young (y) and elderly (o) animals. The average values of six animals per group are presented in Figure 3.09. Histograms of the number of fibres showed a significant leftward shift in the DEDL of young showing a significant increase in the number of small breadth fibres present (Figure 3.09A, green). A similar response was present in response to denervation present in elderly muscle, which showed a significant increase in the number of small diameter fibres (Figure 3.09C, red). This indicates a decline in fibre breadth between denervated and contralateral innervated limbs regardless of age, as confirmed by a non-parametric Kruskal-Wallis analysis of average fibre breadth following denervation (Figure 3.09B, D). Histogram analysis depicts very similar trends in response between the young and elderly animals. However, in both DEDL and EDL the number of fibres present in the young animals are significantly greater than in the elderly group, a trend not unique to this experiment (Sheard & Anderson, 2012). Young and elderly fibres showed comparable scales of atrophy in response to denervation as no significant differences in fibre breadth were seen following non-parametric Kruskal–Wallis and post-hoc Dunn’s multiple comparison analyses (Figure 3.09F).
Figure 3.09 Fibre size distributions

Histograms compare the average number of fibres present in DEDL and EDL muscles within a defined bin width (2.64 μm) from six young (A), six elderly (C) and combined young and elderly (E). (A, C) A significant leftward shift for the DEDL muscle of both young and elderly compared to EDL. (B, D) A significant reduction in the average DEDL fibre breadths compared to EDL. (E) Histograms of both young and elderly responses to denervation indicate similar responses. (F) No significant differences between the average fibre breadths of the two age groups were present.
As muscle fibre atrophy can also be caused by disuse of muscle fibres it is plausible that surgery may have altered gait and resulted in the shift of fibres size. Therefore, in order to verify that the cause of atrophy was not due to disuse of the limb following surgery, I compared muscle fibre breadth in two soleus muscles, one from the surgically altered limb (SolD) and one from the unaltered limb (SolI) from the same animal. The comparison revealed significant increases in fibre breadth in the soleus from the surgically altered limb (Figure 3.10A), verified by an un-paired $t$-test of the two muscle groups (Figure 3.10B).

3.4 Response to surgical denervation in youth

When young muscle fibres are denervated they show a large increase in surface expression of NCAM, especially extra-junctionally (Covault & Sanes, 1985, 1986). I sought to replicate this experiment to determine if the muscle fibres in my mice responded to denervation in the same manner. I was also looking to develop an understanding of specific responses of NCAM as a function of its post-translational modification through the addition of polysialic acid.

3.4.1 Whole NCAM levels

Since the majority of previous work on NCAM in response to denervation examined whole NCAM (unpolysialylated and polysialylated NCAM combined), we deemed it necessary to evaluate these changes in our model of denervation (Covault & Sanes, 1985; Andersson et al., 1993; Walsh et al., 2000). As an indication of relative protein

![Figure 3.10 Soleus fibre size distribution](image)

**Figure 3.10 Soleus fibre size distribution**

Solei from surgically altered limb (SolD) and unaltered limb (SolI) act as a control for disuse atrophy. A) The fibre size distribution of both muscles is depicted by a histogram showing the number of fibres against fibre breadth defined by certain bin widths (2.64 $\mu$m). B) Average fibre breadth of both muscles shows significant increase associated with the surgical limb. Data expressed as mean ± SD, * = p<0.01.
expression, we measured the brightness of immunofluorescence using its mean greyscale value (GV) (0-255) (Methods 2.5.8.3.1). I assessed three groups of specimens for NCAM staining: denervated fibres from the surgical limb (DEDL-D); innervated fibres from the surgical limb (DEDL-I); and innervated fibres from the contralateral limb (EDL-I). The relative fluorescence was measured at junctional and extra-junctional regions of the muscle fibres (Methods 2.5.8.3.2). Statistical analysis was performed using a test for normality using a D'Agostino & Pearson omnibus normality test. As datasets presented as abnormally distributed the data was log transformed. Some datasets remained abnormally distributed and therefore a non-parametric Kruskal–Wallis with post-hoc Dunn's multiple comparison analysis was used to determine differences between the three major variables: denervated NCAM levels, innervated NCAM levels in surgical muscle and innervated NCAM levels in control muscle.

Analysis of the three variables revealed a significant (p<0.05) 16.8% decrease in junctional NCAM in denervated EDL muscle (33.05 GV) relative to innervated EDL muscle (39.74 GV) of the contralateral limb (Figure 3.11A). Extra-junctional NCAM measurements revealed significant 36% increase in denervated fibres (DEDL D, 20.5 GV) from surgical muscle relative to innervated fibres (DEDL I, 13.06 GV). While a 31% increase in NCAM was present from denervated fibres from the denervated muscle relative to innervated fibres of the contralateral limb (EDL I, 14.11 GV). However, there was no significant difference between the internal (DEDL I) and external (EDL I) control muscles extra-junctional NCAM (Figure 3.11B).

![Figure 3.11 Whole NCAM levels](image)

**Figure 3.11 Whole NCAM levels**

Junctional and extra-junctional whole NCAM levels across denervated fibres of the surgically denervated limb (DEDL D), innervated fibres of the surgically denervated limb (DEDL I) and innervated fibres from the contralateral limb (EDL I) of two month old mice. Junctional changes in NCAM expression include a significant reduction in expression from DEDL D to EDL I and from DEDL I to EDL I. Extra-junctional changes include significant increase in NCAM in DEDL D compared to both DEDL I and EDL I. Data expressed as mean ± SD, ** = p<0.01, *** = p<0.001, **** = p<0.0001.
3.4.2 Polysialic acid and NCAM

To further understand the response of muscle, two forms of NCAM were investigated: un-polysialylated NCAM (unNCAM), the inactive form associated with cell adhesion (Rønn et al., 2000), and polysialylated NCAM (PSA-NCAM) which has roles associated with intracellular signalling and regulation of gene expression (Rousselot et al., 1995; Rønn et al., 2002). The responses of both forms were investigated in muscles of six denervated (DEDL) and six control (EDL) muscles.

3.4.2.1 UnNCAM levels

Denervated fibres from the surgical limb (DEDL-D), innervated fibres from the surgical limb (DEDL-I) and innervated fibres from the contralateral control limb (EDL-I) were assessed for unNCAM staining. The relative fluorescence of unNCAM staining was measured at junctional extra-junctional regions of the muscle fibres (Methods 2.5.8.3.2). These values were also tested for normality using a D’Agostino & Pearson omnibus test described in methods 2.5.8.4. Determination of abnormally distributed data allowed non-parametric Kruskal–Wallis with post HOC Dunn’s multiple comparison analysis of the IHC staining (Figure 3.12).

Analysis of the three variables revealed a significant decline of 27.1% in junctional unNCAM in denervated fibres from surgical EDL muscle (29.64 GV) relative to innervated fibres from control EDL muscle (40.63 GV). A significant decline of 18.5% in junctional unNCAM was also present in innervated fibres of surgical muscle (DEDL I, 33.12 GV) relative to innervated fibres from control muscle as seen in Figure 3.12A. Extra-junctional unNCAM measurements revealed a significant 36% increase in unNCAM in denervated fibres from the surgical EDL (DEDL D, 20.5 GV) relative to innervated fibres from the surgical EDL (DEDL I, 13.06 GV). While a 31% increase in unNCAM was present in denervated fibres from the surgical EDL relative to control EDL (EDL I, 14.11 GV). However, there was no significant difference in extra-junctional expression of unNCAM between the internal (DEDL I) and external (EDL I) control muscles (Figure 3.12B).
**Results**

Figure 3.12 Young unNCAM junctional and extra-junctional levels

A and B) Junctional and extra-junctional expression of unNCAM across denervated fibres of the surgical limb (DEDL D), innervated fibres of the surgical limb (DEDL I) and innervated fibres from the contralateral control limb (EDL I) of two-month-old mice. Extra-junctional staining is evident in image C) (DEDL D) (hollow arrow) compared to the junctional staining (solid arrow). Reduced extra-junctional expression in DEDL I (image D) and EDL I (image E) is evident (hollow arrows). Negative control shows no staining (white insert E). Scale 25 μm. Data expressed as mean ± SD, ** = p<0.01, **** = p<0.0001.
3.4.2.2 PSA-NCAM levels

The three young groups of specimens (denervated fibres from the surgical limb; innervated fibres from the surgical limb; and innervated fibres from the contralateral limb) were also measured for PSA-NCAM junctional and cytoplasmic staining (Methods 2.5.8.3.1). Cytoplasmic measurements are important as they indicate the relative intracellular signalling activity of PSA-NCAM and have previously been seen in abundance in the cytoplasm (Kiss et al., 1994). Analysis of PSA-NCAM data followed the methods described in section 2.5.8.4, allowing comparison of IHC staining for each variable (Figure 3.13C, D & E).

Comparison of junctional PSA-NCAM expression revealed no significant differences between the three variables (Figure 3.13A). In contrast, cytoplasmic PSA-NCAM measurements revealed a significant increase of 27% in IHC staining of denervated fibres (DEDL D, 31.66 GV) from the surgical muscle relative to innervated fibres (DEDL I, 23.22 GV). While a 37% increase NCAM was seen in denervated fibres of the surgical muscle relative to innervated fibres of the control EDL muscle (EDL I, 19.85 GV). No significant differences in cytoplasmic expression were present between the two innervated fibre groups (Figure 3.13B).

3.4.3 Summary

The above results are in partial agreement with previous work by Covault and Sanes (1985). Increases in extra-junctional unNCAM expression were seen in response to denervation in young animals but there was also a decline in junctional expression. As seen in unNCAM the youthful response of muscle to denervation did not follow the previously described junctional increase, although unlike unNCAM, PSA-NCAM showed no significant change in expression associated with denervation. However, changes in the cytoplasmic levels of PSA-NCAM increased dramatically in the young animals following denervation.
Figure 3.13 Young PSA-NCAM junctional and cytoplasmic levels

A and B) Junctional and cytoplasmic expression of PSA-NCAM across denervated fibres from the surgically denervated limb (DEDL D), innervated fibres from the surgically denervated limb (DEDL I) and innervated fibres from the contralateral limb control (EDL I) of two-month-old mice. The cytoplasmic staining is evident in image C (DEDL D) (hollow arrow) compared to the junctional staining (solid arrow). Reduced cytoplasmic expression in DEDL I (image D) and EDL I (image C) is evident. Negative control shows no staining (white insert E) Scale 25 μm. Data expressed as mean ± SD, **** = p<0.0001.
3.5 Elderly response to surgical denervation

The impact of denervation on muscles NCAM expressions in elderly animals is yet to be established. Some work has highlighted small reductions in mRNA levels of NCAM and lowered polysialic acid, but little has been done to investigate the resulting changes in protein levels (Andersson et al., 1993; Rutishauser, 2008). This investigation aimed to fill these gaps and further develop an understanding of variations in NCAM and its post-translational modification.

3.5.1 NCAM levels

Both unNCAM and PSA-NCAM were investigated in the elderly model. Their responses in denervated (DEDL) and control (EDL) muscles of six elderly mice were measured junctionally, extra-junctionally and within the cytoplasm.

3.5.1.1 UnNCAM levels

The relative protein levels were deduced from the fluorescence brightness measured as grey value (GV) (Methods 2.5.8.3). Three groups, denervated fibres from the surgical EDL, innervated fibres from the surgical EDL and innervated fibres from control EDL were evaluated for unNCAM staining at junctional and extra-junctional regions (Methods 2.5.8.3.2) (Figure 3.14). Statistical analysis was performed following the methods described in section 2.5.8.4 allowing comparison of the three groups.

Analysis of junctional unNCAM levels of the three variables revealed a small but significant decline of 20% in junctional unNCAM in denervated fibres from surgical EDL muscle (29.42 GV) in relation to innervated fibres from control EDL muscle (36.58 GV). No significant difference between the junctional unNCAM on innervated fibres from both surgical (32.51 GV) and control muscles were present (Figure 3.14A). Extra-junctional unNCAM measurements revealed no significant differences in unNCAM expression across the three groups (Figure 3.14B).
Figure 3.14 Elderly junctional and extra-junctional unNCAM levels

A and B) Junctional and extra-junctional expression of unNCAM across denervated fibres from the surgically denervated limb (DEDL D), innervated fibres from the surgically denervated limb (DEDL I) and innervated fibres from the contralateral limb (EDL I) of 21-month-old mice. The junctional staining pattern (solid arrow) is evident in the above images for DEDL D (C), DEDL I (D) and EDL I (E) compared to the extra-junctional staining (hollow arrow). Negative control image C (white insert). Scale 25 μm, data expressed as mean ± SD, ** = p<0.01.
3.5.1.2 PSA-NCAM levels

Two fibre categories from elderly muscle were examined, denervated fibres from surgical EDL muscles and innervated fibres from control EDL muscles, and junctional and cytoplasmic PSA-NCAM levels were measured (Figure 3.15). Statistical analysis was performed using $t$-test and Mann-Whitney U techniques described in methods 2.5.8.4 allowing comparisons between the IHC staining of the two major variables.

Only two muscle groups were analysed for PSA-NCAM expression as no innervated fibres from the surgically denervated muscle (DEDL I) were identified. The statistical analysis of the remaining two variables revealed a significant decline of 40% in junctional PSA-NCAM on denervated fibres of surgical EDL muscle (29.22 GV) in relation to innervated fibres of control EDL muscles (49.04 GV) (Figure 3.15A). In comparison, cytoplasmic PSA-NCAM showed no significant difference between denervated fibres from surgical muscles (DEDL D, 27.4 GV) and innervated fibres of control muscles (EDL I, 30.69 GV) (Figure 3.15B).
These results show that the response of elderly muscles to denervation is unlike that of young animals. Changes in unNCAM showed small declines in junctional levels, however, there was no change in extra-junctional unNCAM associated with elderly denervation. The elderly response of muscle to express PSA-NCAM in denervation features a junctional decline comparable to unNCAM, while cytoplasmic levels of PSA-NCAM remained constant.
3.6 NaV$_{1.5}$ expression

Evaluating the efficacy of the NaV$_{1.5}$ antibody as a marker for denervation (Rowan et al., 2012) required determination of the innervation status of the muscle fibres using pre-synaptic terminal marker SYN. In young animals it was identified that in 90% of the fibres investigated and imaged for NCAM analysis the same subset of fibres that were deemed denervated (DEDL D) were also found to have high NaV$_{1.5}$ expression, a finding consistent with that of Rowan et al. (2012) (Figure 3.16C). NaV$_{1.5}$ staining of DEDL D muscle was seen to extend beyond the endplate region of the muscle fibres (Figure 3.16C hollow arrow), as fibres without post-synaptic apparatuses were also immunostained.

![Figure 3.16 Nav1.5 Analysis](image)

Nav1.5 staining present in both young and elderly animals in response to denervation. A) Synaptophysin staining absent (solid arrow) in the DEDL D muscle of a 2 month of animal, in the presence of a post-synaptic apparatus demarcated by α-BTX in image B (solid arrow). C) Nav1.5 staining present at the junction and encircling the denervated fibre (solid arrow). Also depicted is the neighbouring fibre (hollow arrow) that is encircled but does not have a post-synaptic (B, hollow arrow) or pre-synaptic terminal (A, hollow arrow). Negative control showed no staining (image A white insert). Scale 25 μm.
3.7. Elderly vs young comparison

Both young and elderly animals respond to surgical denervation with altered NCAM expression, so it was important to compare the denervated fibres from surgical EDL muscles with innervated fibres from control EDL muscles to evaluate differences between young and elderly mice.

3.7.1 UnNCAM levels

The young and elderly muscle groups from 12 animals were measured for junctional and extra-junctional unNCAM levels in response to denervation (methods 2.5.8.3.1). Statistical analysis was performed via the protocols described in methods 2.5.8.4 to allow age comparisons of IHC expression.

The comparative responses of young and elderly muscle to denervation showed no significant differences in junctional unNCAM. Only within-age (2 months and 21 months) differences between the denervated fibres of surgical EDL muscles and innervated fibres from control EDL muscles were present (Figure 3.17A). Extra-junctional analysis displayed similar unNCAM patterns as significant increases were seen in denervated fibres of surgical EDL muscles relative to innervated fibres of control EDL muscles in both elderly and young groups. In contrast to junctional unNCAM, extra-junctional increases in elderly unNCAM in the denervated fibres from surgical EDL muscles (18.71 GV) were 8.7% less than the increase in denervated fibres in young animals (20.5 GV). Such a decline suggests an age-associated reduction in the ability to up-regulate extra-junctional unNCAM in response to denervation (Figure 3.17B).
3.7.2 PSA-NCAM levels

Junctional and cytoplasmic PSA-NCAM levels in denervated fibres from surgical EDL muscles and innervated fibres from control EDL muscles were compared. Statistical analysis (Methods 2.5.8.4) allowed age comparisons of IHC staining.

Junctional PSA-NCAM revealed a significant decline in elderly denervated fibres in surgical EDL muscle (29.22 GV) relative to innervated fibres in control EDL muscles (49.04 GV). This junctional decline was 22% larger than the decline present in young animals (Figure 3.18A). Comparatively, cytoplasmic PSA-NCAM in young animals showed a significant increase in denervated fibres in surgical EDL (35.34) relative to innervated fibres in control EDL (19.85 GV). However, a substantial 23% smaller response was present in elderly denervated fibres in surgical EDL muscle (27.4 GV).
3.7.3 Summary

Analysis revealed an age-associated alteration in response to denervation. In both unNCAM and PSA-NCAM, elderly denervated muscle fibres showed a denervation response that followed the trend seen in young animals. However, in both extra-junctional unNCAM and cytoplasmic PSA-NCAM the elderly response was significantly reduced compared to young animals.
3.8 Protein per pixel and area analysis

The immunofluorescent area was determined by measuring the muscle surface occupied by staining (Methods 2.5.8.3). I estimated protein per pixel (PPP) by dividing the relative fluorescence value by the immunofluorescent area occupied. This represented the dispersal of NCAM expression and allowed comparison between young and elderly muscle fibres. This was potentially important because denervated fibres undergo atrophy, reducing cytoplasmic volume and fibre surface area. The relative protein levels were deduced from the relative fluorescent brightness measured as grey value. Denervated muscle fibres from surgically denervated EDL (DEDL D) and innervated muscle fibres from control EDL (EDL I) of six young and six elderly animals were examined.

3.8.1 UnNCAM levels

3.8.1.1 Junctional data

A comparison of the PPP between young (y) and elderly (o) showed no significant differences across the four groups (Figure 3.19). A declining trend in young junctional PPP and area were present from innervated fibres of control EDL (yEDL I, 0.088 PPP) to denervated muscle fibres of surgical EDL muscles (yDEDL D, 0.065 PPP). Contrasting results were present in elderly animals as both PPP and area measurements remained constant following denervation.

**Figure 3.19 PPP and area comparison of junctional unNCAM levels**

Comparison of young and elderly junctional protein per pixel (PPP) (A) and area of fluorescent staining per pixel (area (pixels)) (B). PPP showed no significant difference between the DEDL D and EDL I groups of both young and elderly junctional unNCAM expression. Area of fluorescent staining showed no significant trend of decline in both young and elderly groups from EDL I to DEDL D. Data expressed as mean ± SD.
3.8.1.2 Extra-junctional data

Elderly denervated fibres showed significantly higher extra-junctional PPP (0.031) compared to young denervated fibres (0.021 PPP) (Figure 3.20A). However, the area occupied by IHC staining showed large age-related reductions from young (1258 pixels) to elderly (679.6 pixels) (Figure 3.20B). This suggests higher elderly protein concentration is a consequence of reduced area as opposed to increased production. In contrast, young denervated fibres showed consistent protein concentration with increasing extra-junctional area.

Figure 3.20 PPP and area comparison of extra-junctional unNCAM levels
Comparison of young and elderly extra-junctional protein per pixel (PPP) (A) and area of fluorescent staining (Area in pixels) (B). PPP showed a significant increase in extra-junctional unNCAM PPP from yDEDL D to oDEDL D. The area of fluorescent staining showed an increasing trend in young when denervated (EDL I tp DEDL D). An age comparison shows a large non-significant increase in extra-junctional area from elderly denervated (oDEDL D) to young denervated fibres (yDEDL D). Data expressed as mean ± SD, *** = p<0.001.

3.8.2 PSA-NCAM levels

3.8.2.1 Junctional data

Junctional PPP and area data revealed no significant differences between the four groups (Figure 3.21). PPP levels were conserved across all four groups potentially as a function of age-related variations in junctional area. Elderly denervated fibres had larger declines in junctional area compared to young animals, suggesting young animals respond to denervation by increasing PSA-NCAM and maintaining junctional concentration. This response is potentially lost in elderly animals where junctional area is reduced resulting in a maintained concentration.
Results

3.8.2.2 Cytoplasmic data

Comparison of young and elderly PSA-NCAM PPP and area revealed consistent declines in area in response to denervation (Figure 3.22). In contrast, young animals featured a denervation associated increase in cytoplasmic concentration. This feature was not present in the elderly animals, suggesting an age-related decline in PSA-NCAM production in response to denervation in spite of a significantly higher cytoplasmic concentration.

Figure 3.22 PPP and area comparison of cytoplasmic PSA-NCAM levels
Age comparison of protein per pixel (PPP) expression of PSA-NCAM and overall area of fluorescent staining. (A) PPP showed a significant increase from young EDL I to DEDL D. (B) Elderly fibres show a trending decline from EDL I to DEDL D. The area of fluorescent staining of both young and elderly cytoplasmic PSA-NCAM expression showed trending declines in response to denervation from EDL I to DEDL D. Data expressed as mean ± SD, ** = p<0.01, **** = p<0.0001.
4. CHAPTER FOUR
DISCUSSION
4.1 Overview

Sarcopenia manifests as the loss of skeletal muscle mass as a consequence of myofibre atrophy or death (Mitchell et al., 2012). The cause of myofibre atrophy is still controversial but a prominent current hypothesis suggests motoneurons ($\alpha$-MN) driving large, fast motor units seem differentially susceptible to premature death (Campbell et al., 1973; Tomlinson & Irving, 1977; Lexell, 1997; Cruz-Sanchez et al., 1998; McNeil et al., 2005). Their death leaves seemingly healthy muscle fibres denervated which stimulates them to produce a “neurite attracting factor” (NAF) to promote re-innervation. Nerve sprouts grow to re- innervate denervated fibres with several structural and functional consequences potentially including fibre type transformation and fibre type clumping. However, with advancing age some denervated fibres fail to become re-innervated by neighbouring nerve sprouts resulting in fibres that remain denervated in the long term and which then undergo atrophy (Viguie et al., 1997; Rowan et al., 2012). It is the presence of these long-term denervated fibres that motivates this investigation and the question, why do elderly muscle fibres fail to become re-innervated?

One candidate molecule for the NAF is Neural Cell Adhesion Molecule (NCAM), due to its involvement in nerve growth during development, down-regulation in maturity for synaptic maintainence (except in highly plastic brain areas) and denervation stimulated up-regulation in the muscles of young animals (Covault & Sanes, 1985; Angata & Fukuda, 2003). The purpose of this investigation therefore, was to quantify and describe the changes in young and elderly muscle NCAM levels, in response to denervation. I proposed that our model of denervation would replicate changes in NCAM levels previously seen in young animals following denervation, and that changes in the levels of each isoform would relate to their proposed functions. I also proposed that elderly muscles would show an impaired response to denervation and that this may underpin the failure of sarcopenic muscle to become reinervated.

I was able to replicate previous findings showing that young mouse muscle responds to denervation with an increased production of NCAM. The cell adhesion associated form (un-polysialylated-NCAM) showed large extra-junctional increases and the intracellular signalling form (polysialylated-NCAM) also showed cytoplasmic increases in young
animals. Although Covault and Sanes (1986) did not discriminate between isoforms, this finding is largely in agreement with theirs. By contrast, elderly mice did not respond to denervation with an increase in NCAM isoforms. This main finding is consistent with my hypothesis that impaired ability to re-attract a nerve sprout by release of a diffusible signal may contribute to long-term denervation in elderly muscles. Herein, I will describe how these finding inform our current understanding of sarcopenic pathophysiological mechanisms.

4.2 Methodology

To fully understand NCAM and how muscle responds to denervation within both the young and elderly murine models, I first had to design an appropriate protocol for denervation. Previous investigations focus on whole limb (Covault & Sanes, 1985; Walsh et al., 2000) or whole muscle denervation (Covault & Sanes, 1986) (Figure 4.01A). I modified a whole muscle protocol and selected the extensor digitorum longus muscle (EDL) for study as the deep peroneal nerve bifurcates on entry into the muscle allowing for a partial denervation (Figure 4.01B). Utilising a partial denervation has minimal effect on muscle function, meaning that any associated changes in NCAM expression can be separated from widespread disuse and gait alteration. A partial denervation also provides an internal control as both denervated and innervated muscle fibres are present within the muscle, and the contralateral EDL muscle can be used as an intact control.

**Figure 4.01 Extensor digitorum longus innervation**

Figure depicts the innervation pattern of extensor digitorum longus muscle from the deep peroneal nerve. A) Depicts the crush completed by (Covault & Sanes, 1985) to create a whole muscle denervation. B) Depicts the branch crush used in the current experiment to create a partial crush. Adapted and modified from Balice-Gordon & Thompson, 1988.
The first difficulty associated with performing a partial denervation was the size of the nerve as the bifurcated nerve is only 50 μm in diameter (Figure 2.01). Therefore, confirmation that the crush severed motor axons to EDL was crucial, so I explored several validation techniques (Methods 2.5.5). The use of DiO neurotracer was ineffective as the small nerve surface area made it difficult to introduce enough neurotracer into the nerve for effective labelling. The tissue clearing techniques investigated also failed to allow adequate nerve visualisation due to high levels of autofluorescence. The BABB protocol (Methods 2.5.5.1) involved dehydration of the tissue in methanol and resulted in fluorescent quenching and a reduced signal-to-noise ratio making it difficult to discern anti-neurofilament staining (Ke et al., 2013). The Scale protocol (Methods 2.5.5.2) was also ineffective as the technique was too slow (Hama et al., 2011; Ke et al., 2013). Instead the efficacy of the crush was determined by immunohistochemistry (IHC) on serial sections, using an antibody to synaptophysin (SYN) (a pre-synaptic terminal marker), fluorescent α-bungarotoxin (a post-synaptic terminal marker) and an antibody to voltage-gated sodium channel 1.5 (NaV1.5) (a marker for denervation) (Yang et al., 1991; Rowan et al., 2012). Using this protocol I was able to confirm that a subset of fibres within the experimental muscles were denervated. Muscles showed significant fibre atrophy (Figure 3.8C/D) compared to the external controls (Figure 3.09E/F), a change not due to disuse as shown by the control muscle comparison (Figure 3.09). Denervated NMJ presented without pre-synaptic terminal labeling in the presence of α-BTX (Figure 3.8E, F &G) on fibres encircled with NaV1.5 staining (Figure 3.15). All of these factors show that our model of denervation was experimentally sound and that changes in the different forms of NCAM and levels measured in such fibres are a reflection of events occurring in response to denervation.

4.3 Muscle response to denervation

One of the major responses of skeletal muscle to denervation is the up-regulation of genes associated with a variety of processes required for myofibre survival: synaptic signallling receptor activity regulated by modifying protein 1 and calcitonin-receptor-like receptor (Ramp-1 & crlr); protection against myofibre atrophy initiated by runt-related transcription factor-1 (Runx-1); and stimulation of re-innervation by NCAM (NCAM1) (Barns et al., 2014). The NCAM1 gene which signals for the production of
NCAM is thought to be up-regulated in the muscle's endeavour to attract a neighbouring nerve sprout (Covault et al., 1986; Covault & Sanes, 1986; Andersson et al., 1993).

**4.3.1 Changes in NCAM levels in young**

In young animals following denervation NCAM levels is maintained at junctional areas but shows significant increases in both mRNA and protein levels at extra-junctional areas of the muscle (Covault & Sanes, 1985; Andersson et al., 1993; Walsh et al., 2000). The current investigation attempted to repeat this work with our IHC assay. We established that whole NCAM levels (combination of both polysialylated and unpolysialylated) in young animals showed slight reductions at the NMJ coupled with large increases extra-junctionally following denervation (Results 3.4.1.1). The outcome might be described as a flattening of the normal distribution of NCAM which is normally at high levels junctionally and low levels extra-junctionally (Figure 4.02).

![Figure 4.02 Schematic showing NCAM expression across muscle surface](image)

The distribution of whole NCAM expression across the muscle surface is categorised as junctional (striped) or extra-junctional (solid) levels. The expression of NCAM distribution changes from innervated (blue striped) to denervated (red striped) with an increase in extra-junctional expression (red solid) and small decrease in junctional expression (red striped) associated with denervation of a muscle fibre.

Such a pattern of expression suggests NCAM production does not dramatically increase in response to denervation but instead distribution is altered. Together these results suggest that in response to denervation, muscles in young animals’ up-regulate and redirect NCAM from the junctional folds to the extra-junctional portion of the muscle thereby increasing surface area over which NCAM is present with a consequential increase in the chance of attracting a neighbouring nerve sprout for re-innervation. As
previously discussed (Introduction 1.09), denervated muscle fibres are able to become re-innervated by neighbouring nerve sprouts from up to 200 µm away. Increasing the extra-junctional area for attractant release may expand the field of attraction and the probability of becoming re-innervated (Figure 4.03).

To be effective away from the denervated fibre, the attractant cannot be membrane-bound. Recently the role of a post-translational modification involving the addition of polysialic acid (PSA) to NCAM was discovered. PSA was shown to act as a steric hindrance molecule preventing NCAM acting as a cell adhesion molecule and instead activating intracellular signalling properties involved in calcium handling and gene expression (Walsh & Doherty, 1991; Bassel-Duby & Olson, 2006). The addition of PSA has also been linked to the release of a soluble form of NCAM which activates calcium signalling and gene expression in neighbouring cells (Rousselot et al., 1995; Rutishauser, 2008). I propose that such a diffusible modified form of PSA-NCAM may be the NAF released by denervated fibres. For this reason the potential roles of both forms of NCAM (polysialylated-NCAM and unpolyisialylated-NCAM) in response to denervation required consideration.
4.3.2 Influence of polysialic acid

To expand on the notion that PSA plays a vital role in the regulation of NCAM's properties (Olsen et al., 1995; Rutishauser, 2008), this investigation looked at the differences in the levels of modified and unmodified forms of NCAM in relation to denervation. The unNCAM findings reflected previously described results from whole muscle NCAM measurements, with increases of 18.5% associated with denervation of the muscle compared to its external control (Covault & Sanes, 1985). The large extra-junctional increases in NCAM may be associated with increasing the attraction area for re-innervation. In addition, since unNCAM is associated with cell adhesion, increasing the extra-junctional levels potentially increases the anchorage points for nerve sprouts that are able to locate and innervate the denervated myofibres.

PSA-NCAM showed no significant changes in junctional levels between denervated and innervated muscles. However, cytoplasmic staining of PSA-NCAM revealed a 37.3% higher level compared to innervated external controls. This substantial increase suggests that large amounts of PSA-NCAM are being synthesised and shuttled to the fibre surface. Subsequent activation of membrane bound PSA-NCAM is vital for intracellular signalling pathways associated with calcium handling, gene expression and cleavage as well as release of soluble NCAM that may act as the NAF to attract neighbouring nerve sprouts (Walsh & Doherty, 1991; Kiselyov et al., 2005; Secher, 2010).

4.3.3 Elderly response to denervation

To truly understand the role of NCAM in skeletal muscle innervation and denervation we need to understand what occurs in the denervated pathophysiological state in elderly mice. Changes that occur in elderly muscles include autophagic dysfunction, neuromuscular fragmentation (Li et al., 2011; Carnio et al., 2014), oxidative damage due to mitochondrial dysregulation and the appearance of atrophic and dying myofibres (Cuervo et al., 2005).

Quantification of the comparative changes of unNCAM and PSA-NCAM between young and elderly animals revealed novel findings in response to denervation. Although young and elderly animals showed comparable trends in junctional declines and extra-junctional increases of unNCAM levels, the extra-junctional increase in unNCAM was
8.7% less (p<0.05) in elderly animals than in their younger counterparts. This novel outcome is consistent with the hypothesis that an age-related reduction in muscle production of NCAM exists, but furthermore highlights that elderly animals may possess a reduced ability to redirect unNCAM in response to denervation. Such a decline in unNCAM availability may reduce the capacity to anchor neighbouring nerve sprouts in areas outside the junctional region, in turn reducing the chance of re-innervation.

In contrast, the abundance of PSA-NCAM in muscle did not show comparable trends between young and elderly animals in response to denervation, as junctional PSA-NCAM remained constant in the young animals. This response was not seen in the elderly animals which showed significant junctional declines following denervation. The trend continued with cytoplasmic PSA-NCAM levels, as young animals demonstrated increases following denervation whereas elderly animals had 22.5% less PSA-NCAM. Such reductions correlate with previously described PSA synthesis in elderly animals (Olsen et al., 1995). As PSA-NCAM may normally act to create an attractant gradient for neighbouring motoneurons to use as a pathway to denervated muscle, a decrease in PSA-NCAM may severely hinder the ability of elderly animals to attract neural input by reducing activation of intracellular pathways involved in releasing cleaved NCAM (Walmod et al., 2004; Secher, 2010). Therefore, the levels of NCAM present in the surrounding ECM are reduced hindering the ability of muscle to attract a neural input by reducing the effective area of attraction that may be present in young animals in response to denervation (Figure 4.03).

The current results show a reduced capacity to produce both forms of NCAM in response to denervation in the elderly animals. This is not in agreement with previous measurements of NCAM mRNA levels which show an up-regulated response in elderly and denervated animals (Covault et al., 1986; Andersson et al., 1993), suggesting the decline in protein may reflect an inability to correctly distribute and traffic NCAM. The current investigation therefore looked to further consider the distribution of the NCAM isoforms within muscle by considering the relative fluorescence per pixel measured (protein per unit area) in the muscle fibres of innervated and denervated young and elderly mice. The data revealed trending increases in junctional and extra-junctional elderly unNCAM protein per pixel (PPP) ratio compared to younger animals even with comparative changes in protein levels between both groups. The elderly increase in PPP
exists as a result of large declines in junctional and extra-junctional areas caused by fibre atrophy. In contrast PSA-NCAM PPP ratios show reductions in junctional and cytoplasmic levels in elderly animals compared to younger animals. PPP reductions were present even with the diminished junctional and cytoplasmic area of the elderly, consistent with the previously mentioned reduction in elderly PSA and NCAM production within myofibres (Olsen et al., 1995). In contrast to the stereotypical response of young muscles to increased NCAM production following denervation, the ability of elderly muscles to respond was drastically reduced, potentially resulting in a diminished ability to both re-distribute unNCAM to extra-junctional areas, and drive PSA-NCAMs intracellular signalling pathways essential for re-innervation. A summary of all changes relative to the young innervated animals is presented below in Table 4.01.

4.4 Muscle or nerve?

Although the current investigation highlights a declining response in the muscle’s production of unNCAM extrajunctionally and PSA-NCAM cytoplasmically following denervation, the magnitude of change apparent may not completely account for the lost re-innervative ability that is a feature of sarcopenia. It is prudent to ask whether loss of re-innervation is in fact an inability of skeletal muscle to produce a NAF (NCAM) in response to denervation, or is it an inability of elderly neurons to correctly respond to such a signal? This investigation focused on the muscle’s NCAM levels by ensuring permanent denervation using a double ligation (Methods 2.4.7). Permanent denervation prevented assessment of the re-innervative response between elderly and young animals. However, previous investigations have looked at elderly animal’s ability to sprout and re-innervate with age (Pestronk et al., 1980) and have shown that although slowed, motoneurons still possess the ability to sprout towards and re-innervate denervated skeletal muscle (Pestronk et al., 1980; Vaughan, 1992; Verdú et al., 2000) (Introduction 1.09). That being the case it suggests that the main problem lies with the skeletal muscles ability to stimulate a sprouting response. With such important roles in synaptic regulation and attraction, any age-related decline in PSA-NCAM in response to denervation may have a major influence on muscle’s ability to attract a sprout. This begs the question, what is happening to the regulation of NCAM in elderly skeletal muscle?
<table>
<thead>
<tr>
<th>Animal</th>
<th>Whole NCAM</th>
<th>mRNA NCAM</th>
<th>UnNCAM</th>
<th>PSA-NCAM</th>
<th>PSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Junctional</td>
<td>Extra-junctional</td>
<td>Junctional</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Protein</td>
<td>Area</td>
<td>Protein</td>
</tr>
<tr>
<td>Young innervated (YI)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Young denervated (YD)</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>Elderly innervated (EI)</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td>23% less</td>
<td>↑</td>
</tr>
<tr>
<td>Elderly Denervated (ED)</td>
<td>↑</td>
<td>12-15 fold</td>
<td>Equal</td>
<td>19% less</td>
<td>9% less</td>
</tr>
<tr>
<td>References</td>
<td>(Covault &amp; Sanes, 1985; Kohayashi et al., 1992)</td>
<td>(Covault et al., 1986)</td>
<td>(Olsen et al., 1995)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The table above shows a summary of the changes in NCAM levels present between young and elderly mice based on innervation status. All arrow sizes indicate the magnitude of change present relative to the baseline values of young innervated animal’s measurements (small = slight change, large = big change). Red writing represents changes relative to young denervated animals.
4.5 Possible mechanisms for NCAM dysregulation

The current investigation highlights a reduced unNCAM and PSA-NCAM production in response to denervation in elderly animals. Age-related denervation may be coupled to either an inability to correctly synthesise NCAM from its constituent mRNA, a misfolding of NCAM, or deregulated trafficking to its required junctional and extra-junctional locations.

4.5.1 Gene expression

In order to attribute the decline in NCAM protein levels to decreased gene transcription, we would have to assume that all NCAM1 mRNA gets translated into NCAM proteins. However, this cascade is not trivial and requires a number of steps and processes to work optimally (Covault et al., 1986). To synthesise protein from mRNA the full mRNA sequence is required, the correct post-transcriptional modification (exon removal) must be completed and the strand must be correctly shuttled from the nucleus to the cytoplasm for translation by a ribosome (McCarthy & Gualerzi, 1990; Lewin et al., 2011). The potential for dysregulation increases with so many steps, and may underpin the changes observed in this investigation. Therefore to assess whether the previously described up-regulation of mRNA expression in elderly animals results in synthesised protein, investigation of microRNA (miRNA) levels needs to be conducted (Figure 4.06a). MiRNA are small non-coding double stranded RNAs, approximately 19 -24 nucleotides in length (Purvis et al., 2015). MiRNAs regulate the coding of mRNAs/protein levels either by up-regulating or down-regulating their translation. MiRNAs can then be detected in blood plasma using quantitative Polymerase Chain Reaction at a more reliable level than that of the protein levels, in this case potentially allowing determination of the presence or absence of NCAM production in response to denervation (Purvis et al., 2015).

Another possible scenario is an error in protein folding (misfolding). Misfolding is commonly associated with ageing as a result of increased levels of reactive oxygen species (ROS) that disrupt the protein production pathway (McArdle et al., 2002; Hipkiss, 2006). The resultant misfolded proteins are then targeted for degradation via autophagic pathways. An upregulation of NCAM mRNA expression in the muscles of elderly mice (Covault et al., 1986) in response to denervation may result in increased
production of misfolded NCAM protein destined for degradation. This degradation would account for the reduced response to denervation that is seen in elderly skeletal muscle. Up-regulation and accumulation of misfolded proteins within the cytoplasm can result in plaque formation before the proteins are targeted to degradation (Hipkiss, 2006). Localisation of such plaques with NCAM IHC would potentially ascertain the presence or absence of misfolded protein (Figure 4.06b) (Fukuchi et al., 1998). This could be examined in future to see whether it is at work in elderly denervated fibres.

4.5.2 NCAM trafficking

If I were to assume that the mRNA up-regulation was to result in an increase in protein production, it must then be shuttled to the surface for expression by the process known as exocytosis. Exocytosis is the process by which proteins synthesised in the cytoplasm and packaged within secretory vesicles at the Golgi apparatus are trafficked to the surface for release. Within neurons there are two main types of secretory vesicle, synaptic vesicles (SV) which carry neurotransmitters, and large dense core vesicles (LV) which transport neuropeptides. In the case of skeletal muscle there is a third vesicuotubular organelle that uses a microtubule-dependent mechanism to move vesicles from the trans face of the Golgi apparatus to the cell surface (Ninomiya et al., 1996; Marchand et al., 2002). The trafficking of secretory vesicles containing proteins including NCAM can occur one of two ways. First, though an activity dependent pathway in which the protein is stored in vesicles at a pre-determined location within the cell waiting for a signal allowing release to the membrane. Neurons and β islets of the pancreas are believed to use this form of activity-dependent exocytosis as calcium currents regulate the cell activity and determine release of NCAM (Kiss et al., 1994; Ninomiya et al., 1996; Hatakeyama et al., 2007). Second, vesicle release may be via the constitutive pathway in which protein is shuttled to the surface upon synthesis. Constitutive exocytosis is the process believed to occur in hypothalamic neurons and astrocytes (Pierre et al., 2001) (Figure 4.04).
In skeletal muscle NCAM trafficking via regulated or activity-dependent modes would require NCAM to be produced and kept within a compartment of the cell within storage granules. However, upon visualisation of the cytoplasmic staining pattern of PSA-NCAM, it appears to be a diffuse pattern rather than granular (Figure 4.5). Such a pattern of production suggests a constitutive path of protein shuttling to the surface, as it is produced and packaged in small vesicles and transported directly to the surface. The constitutive pathway fits with the regulation of mRNA expression as investigations have shown that the levels are not constant throughout life but instead are up- and down-regulated in response to activity, suggesting that there is an inactivity sensor present in skeletal muscle (Covault et al., 1986; Andersson et al., 1993).
4.5.2.1 Inactivity sensor

The role and presence of an activity sensor within skeletal muscle has been well established. It is thought to involve regulation of gene expression in response to activity driven inwardly moving calcium. The major source of Ca$^{2+}$ entry in skeletal muscle is through voltage-sensitive L-type Ca$^{2+}$ channels, a channel formed of five subunits, one of which is the dihydropyridine receptor ($\alpha_{1S}$), a transmembrane protein containing a Ca$^{2+}$ selective pore and voltage sensor loop. Dihydropyridine receptors, as well as being present at the sarcolemma, are located at the T-tubule triads of the sarcoplasmic reticulum (SR) within skeletal muscle. The Dihydropyridine receptor's close association with the SR is believed to promote an interaction with the ryanodine receptor (RyR1), a regulator of intracellular Ca$^{2+}$ release and skeletal muscle excitation-contraction coupling (Piétri-Rouxel et al., 2009). The importance of the $\alpha_{1S}$ receptor in skeletal muscle activity sensing is highlighted in experiments utilising exon skipping, which allows the down-regulation of $\alpha_{1S}$ expression without inducing lethality during

\textbf{Figure 4.05 Diffuse PSA-NCAM pattern}

Fluorescent PSA-NCAM present diffusely throughout the cytoplasm of a subset of denervated myofibres from the surgical limb of a young C57/B6j mouse (white arrows). Scale 50 μm
development. The localised down-regulation of α1S did not affect the muscle overall
tetanic contraction, however the down regulation stimulated massive muscle atrophy
which often correlated with changes in MHC phenotype (Piétri-Rouzel et al., 2009).
Such changes in the muscle phenotype suggest that α1S has important links with
transcriptional activity (Wheeler et al., 2008). This regulation acts through the α1S
receptor, and the influx of Ca^{2+} activates a series of intracellular pathways including the
family of calmodulin (CaM) binding proteins (consists of Ca^{2+} binding EF hands)
(Wheeler et al., 2008). Ca^{2+} bound CaM undergoes a conformational change, allowing it
to act as an intermediate protein in downstream signalling processes that regulate
transcription, particularly in the activation of kinases, for example calmodulin kinase
(CaMKII) (Chin, 2005). It is the CaMKII activation which regulates intracellular
pathways involved in CREB/MAPK regulation of gene expression (Bhalla & Iyengar,
1999).

Although the activity sensor is well established, an understanding of how this regulates
gene expression in response to inactivity is yet to be discovered but is essential, as a
large number of skeletal muscle associated genes are up- or down-regulated in response
to denervation including: myoD1, myogenin, acetylcholine receptor subunits, Src
homology 2 domain containing protein tyrosine phosphatase substrate 1 (SHPS-1) c-Jun
and c-Fos and NCAM (Covault et al., 1986; Eftimie et al., 1991; Neville et al., 1992;
Weis, 1994; Mitsuhashi et al., 2005). Such a larger number of proteins suggests a
disinhibition of gene transcription associated with reduced activity, or an inactivity-
stimulated pathway might also underpin the change in expression of such proteins.

### 4.5.3 Proteolytic pathway

If NCAM synthesis is increased in response to inactivity or due to a blockade of an
activity sensor, the shuttling of NCAM to the surface using constitutive mechanisms may
be vulnerable to degradation as a result of a dysfunctional proteolytic pathway. The
main pathway involved in membrane protein degradation is the ubiquitin-proteosome
pathway (UPP) (Rubinsztein, 2006; Mizushima & Komatsu, 2011). The importance of
UPP in sarcopenia is derived from its control of two important destabilising factors; the
removal of reactive oxygen species and recycling of misfolded proteins (Carnio et al.,
2014). Ubiquitin mediated proteolysis involves marking a protein for degradation by
cova lent linkage of the small protein cofactor, ubiquitin. Ubiquitin activation occurs
Discussion

through its conversion to a thiol ester by an ATP-requiring enzyme, E1. Activated ubiquitin is then transferred by E1 to one of a family of carrier proteins called E2 proteins, and ubiquitin is coupled by ubiquitin–protein ligase E3 to the protein marked for degradation. The ubiquitin-conjugation reactions are repeated, forming a chain of five or more ubiquitins linked to each other and then to the protein. This modification of the substrate leads to its rapid degradation by a very large proteolytic complex, the 26S proteasome, which requires ATP to function (Mitch & Goldberg, 1996). Age-related changes in the UPP have been reported to occur, of interest is the denervation related up-regulation of UPP hypothesised to drive sarcopenic atrophy through the activation of key regulatory genes MuRF1 and MAFbx (Rowan et al., 2012). If this age- and denervation-stimulated increase in UPP activation targeted NCAM, this would result in the decreased membrane transport hypothesised to cause the reduced levels found in the current investigation. To investigate this, further research would involve blockade of the autophagic pathway using one of four lysosomotropic/protease inhibiting substances: bafilomycin, hydroxychloroquine, chloroquine and ammonium chloride (De Duve et al., 1974; Thiele et al., 1983; Rubinsztein et al., 2007). Lysosomotropic agents inhibit autophagy by altering the degradation of proteins through shear bulk, alteration of pH (enzyme activity) or inhibition of ATPase activity of the autophagic pathway, which results in proteins accumulating in the lysosome (De Duve et al., 1974; Rubinsztein et al., 2007). I propose that mistargeted protein degradation results in the reductions of NCAM present in elderly myofibres. Therefore, lysosomotropic agents could be used to prevent lysosome degradation of NCAM. Prevention of lysosome degradation would allow the presence or absence of NCAM accumulation granules in the cytoplasm to be investigated. The presence of NCAM granules would identify autophagic degradation as a deregulated pathway of age that may be preventing the innate re-innervative ability of myofibres (Figure 4.06C).
Figure 4.06 Understanding reduced NCAM expression

The above flow chart depicts the possible reasons for the reductions in NCAM expression that is seen in elderly animals in response to denervation (green). Yellow boxes represent known results, blue boxes represent hypotheses for the cause of sarcopenia (black box) and red boxes represent methods for evaluating each hypothesis.
4.6 Summary of outcomes

Investigation of the influence denervation has on the expression of NCAM and its forms in young and elderly animals has replicated findings of earlier work and extended those to show that elderly muscle fibres have a different response to denervation than young fibres. The changes associated with young animals supported my hypothesis as they featured up-regulation of both unNCAM extra-junctionally and PSA-NCAM within the cytoplasm. Elevation of NCAM levels in response to a loss of neural input is consistent with control of NCAM expression being regulated by a muscle activity sensor. The blunted response by elderly fibres shows that age alters the ability of muscle to respond to denervation with the same efficacy as in young animals. Other work indicates that elderly mice are capable of regrowth, so the interpretation of the current result is that the presence of long-term denervated fibres in elderly muscles primarily reflects a decreased ability of the muscle fibres to attract neural input. The influence of PSA-NCAM and the effects caused by declining levels in elderly animals is emphasised when considering its roles in intracellular signalling pathways, and its regulation of soluble NCAM release into the ECM as a NAF.

4.7 Conclusions

In an effort to understand the pathophysiological manifestation of the sarcopenic phenotype, I aimed to determine whether the previously described innate ability of young animals to revert to the immature phenotype and express NCAM in response to denervation was altered when considering the presence or absence of polysialic acid. I also aimed to determine whether this ability was lost or altered in elderly animals. My hypothesis was that the changes associated with NCAM would reflect their actions as adhesion molecules and intracellular messengers (unNCAM and PSA-NCAM respectively). I also hypothesised that age-related changes in the expression of NCAM would alter the ability of elderly animals to become re-innervated and in turn contribute to the permanent denervation associated with sarcopenia and muscle atrophy.

The observation of changing NCAM levels at junctional, extra-junctional and cytoplasmic areas revealed a correlation between age, denervation status and declining NCAM levels. I propose that these variations in NCAM levels result from an altered
trafficking pattern associated with elderly animals. To further support this hypothesis, investigations into the alterations of NCAM in sarcopenic tissue is required and to further investigate the means by which NCAM production is inhibited in the elderly system (Figure 4.06). Understanding the mechanisms preventing the re-innervative ability associated with age is imperative for understanding sarcopenia.

4.8 Limitations

The main limitation of the study was associated with access to animals, and an incident with the freezer used for sample storage. Only a relatively small number of animals is available for study at the University of Otago, with very low numbers of elderly males being available. As a result only female mice were used in this experiment and consequently gender specific results could not be acquired even though there are known gender specific differences in susceptibility to age related changes in muscle.

All of the work was from 21-month-old animals, an age which is equivalent to a 63-year-old human. This is at a relatively early stage in the progression of sarcopenic change. Previous work has suggested that the co-morbidities associated with age occur later in C57/B6j mice (>25 months) (Ballak et al., 2014) compared to humans, so that the changes observed might be expected to increase in severity in older mice (+24 months). Increasing severity of age-related changes correlates with investigations showing large changes in protein and mRNA expression of essential maintenance protein, muscle fibre atrophy and death being greatly advanced at 24 months onwards (Ballak et al., 2014; Barns et al., 2014; Gonzalez-Freire et al., 2014). Therefore, similar investigation in older animals may be worthwhile and might show more dramatic changes.

In the 9th month of the investigation before a small final experiment into the effects of NCAM regulation in naturally denervated muscle (sarcopenic tissue) could be conducted we experienced a freezer failure. The resulting thaw increased levels of autofluoresence in tissue that masked the NCAM staining, coupled with the fact that this test experiment used the remaining NCAM anti-body resulting in a small but interesting question not being answered in the current thesis. However further work will be
conducted to investigate these changes to determine whether trends present in the elderly surgical model are also apparent naturally.

The last limitation was the difficulty in clearly establishing a confirmed nerve crush. The first run of surgeries completed included 24 animals, 12 surgical and 12 sham controls. The animals were all run together to rule out any day-to-day variation in immunohistochemical processing. It was found however that the crushes were incomplete and similar numbers of elderly mice were not available to repeat the protocol. The results from the incomplete crushes are not presented in this thesis; except the soleus muscles from the surgical animals were used as sham controls to rule out any associated effects of surgery.
REFERENCES


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Lexell J, Downham D & Sjöström M. (1986). Distribution of different fibre types in human skeletal muscles: Fibre type arrangement in m. vastus lateralis from three groups of healthy men between 15 and 83 years. Journal of the Neurological Sciences 72, 211-222.


6. CHAPTER SIX
APPENDICES
6.1 Reagents Lists

**Benzyl Alcohol Benzyl Benzoate (BABB)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol (50%)</td>
<td>5 mL</td>
</tr>
<tr>
<td>Methanol (100%)</td>
<td>10 mL</td>
</tr>
<tr>
<td>Distilled water (50%)</td>
<td>5 mL</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>1 mL</td>
</tr>
<tr>
<td>Benzyl Benzoate</td>
<td>2 mL</td>
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**Bouins fixative solution**

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<tr>
<td>Saturate Picric acid</td>
<td>300 mL</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>100 mL</td>
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<td>Glacial Acetic Acid</td>
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**Choline esterase stain**

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</thead>
<tbody>
<tr>
<td>Maleic Acid</td>
<td>HO_2CCHCHCO_2H</td>
<td>9.44 mmol.L(^{-1})</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>NaOH</td>
<td>14.7 mmol.L(^{-1})</td>
</tr>
<tr>
<td>Trisodium Citrate</td>
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<td>1.50 mmol.L(^{-1})</td>
</tr>
<tr>
<td>Copper Sulfate</td>
<td>CuSO_4.5H_2O</td>
<td>0.901 mmol.L(^{-1})</td>
</tr>
<tr>
<td>Potassium Ferricyanide</td>
<td>K_3[Fe(CN)_6]</td>
<td>0.155 mmol.L(^{-1})</td>
</tr>
<tr>
<td>Acetylthiocholine</td>
<td>CH(_3)COSCH(_2)CH(_2)N(CH(_3))(_3)I</td>
<td>0.00017 mmol.L(^{-1})</td>
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**Heparinised saline solution**

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<td>Phosphate Buffered Saline</td>
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Permeabilization solution

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Paraformaldehyde solution (1%)

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<td>0.2 L</td>
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<tr>
<td>Paraformaldehyde</td>
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<td>Sodium dihydrogen phosphate</td>
<td>NaH$_2$PO$_4$</td>
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</tr>
<tr>
<td>Disodium hydrogen orthophosphate</td>
<td>Na$_2$HPO$_4$</td>
<td>15.4 mmol.L$^{-1}$</td>
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Phosphate Buffered Saline (1× PBS)

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</tr>
<tr>
<td>Sodium Chloride</td>
<td>NaCl</td>
<td>8 g.L$^{-1}$</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>KCl</td>
<td>0.2 g.L$^{-1}$</td>
</tr>
<tr>
<td>Disodium hydrogen orthophosphate</td>
<td>Na$_2$HPO$_4$</td>
<td>1.44 g.L$^{-1}$</td>
</tr>
<tr>
<td>Potassium hydrogen phosphate</td>
<td>KH$_2$PO$_4$</td>
<td>0.24 g.L$^{-1}$</td>
</tr>
</tbody>
</table>

Saline-Sodium Citrate (SSC)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular formula</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>H$_2$O</td>
<td>1 L</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>NaCl</td>
<td>150 mmol.L$^{-1}$</td>
</tr>
<tr>
<td>Trisodium Citrate</td>
<td>Na$_3$C$_6$H$_5$O$_7$</td>
<td>15 mmol.L$^{-1}$</td>
</tr>
</tbody>
</table>
## Scale A2 clearing agent

Table a6.09 Scale A2 incubation solution

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>4 M</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10%</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.1%</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 mL</td>
</tr>
</tbody>
</table>

## Sodium dodecyl Sulfate (SDS, 0.5%)

Table a6.10 sodium dodecyl sulfate (SDS, 0.5%)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>0.05 g.L-1</td>
</tr>
<tr>
<td>Phosphate buffered saline</td>
<td>100 mL</td>
</tr>
</tbody>
</table>

## Sodium borohydrate (SBH, 1%)

Table a6.11 sodium borohydrate (SBH, 1%)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium borohydrate</td>
<td>0.01 g.L-1</td>
</tr>
<tr>
<td>Phosphate buffered saline</td>
<td>100 mL</td>
</tr>
</tbody>
</table>

## See Deep Brain (SeeDB)

Table a6.12 See Deep Brain (SeeDB)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular formula</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>H₂O</td>
<td>0.1 L</td>
</tr>
<tr>
<td>Fructose (20%)</td>
<td>C₆H₁₂O₆</td>
<td>20 g.wt/vol</td>
</tr>
<tr>
<td>Fructose (40%)</td>
<td>C₆H₁₂O₆</td>
<td>40 g.wt/vol</td>
</tr>
<tr>
<td>Fructose (60%)</td>
<td>C₆H₁₂O₆</td>
<td>60 g.wt/vol</td>
</tr>
<tr>
<td>Fructose (80%)</td>
<td>C₆H₁₂O₆</td>
<td>80 g.wt/vol</td>
</tr>
<tr>
<td>Fructose (100%)</td>
<td>C₆H₁₂O₆</td>
<td>100 g.wt/vol</td>
</tr>
<tr>
<td>SeeDB solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-thioglycerol</td>
<td>HSCH₂CH(OH)CH₂OH</td>
<td>0.5 %</td>
</tr>
</tbody>
</table>

## Tris Buffered Saline (1× PBS)

Table a6.13 Tris Buffered Saline (1× TBS)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular formula</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>H₂O</td>
<td>1 L</td>
</tr>
<tr>
<td>Tris hydrochloride</td>
<td>C₄H₁₂ClNO₃</td>
<td>0.1 M</td>
</tr>
</tbody>
</table>
Tris Buffered Saline (2× PBS)

Table a6.14 Tris Buffered Saline (2× TBS)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular formula</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>H₂O</td>
<td>1 L</td>
</tr>
<tr>
<td>Tris hydrochloride</td>
<td>C₄H₁₂ClNO₃</td>
<td>0.2 M</td>
</tr>
</tbody>
</table>

6.2 Animal dissection protocols

Figure 6.01 Trans-cardial perfusion
This shows the steps involved in perfusion of the mice, following anaesthetisation. A) Toes pinch with forceps to check pedal reflex and depth of anaesthesia. B) Medial incision and dissection of subcutaneous tissue along torso. Bluntly dissecting connective tissue away to allow access to sternum and rib cage. C) Two incisions were made at the sternum, and cuts were made superiorly on either side, towards the clavicle. D) The ribcage was reflected, apex of the heart held to orientate left ventricle, and needle attached to peristaltic pump inserted. E) Right atria was cut and peristaltic pump turned on to deliver heparinised saline and paraformaldehyde.
Figure 6.02 Soleus muscle excision
This shows the steps involved in the removal of the soleus muscle post-fixation. A) The leg is oriented, and the external fascia removed to locate the edge of gastrocnemius. B) reflecting the lateral margin of the gastrocnemius to locate space containing the Soleus muscle. C) Locating, and severing the achilles tendon. D) reflecting the gastrocnemius to reveal the red soleus on its medial surface (black arrow). E) The proximal tendon insertion at the tibia is severed and soleus pulled towards severed achilles tendon (black arrow) to be removed.
Figure 6.05 Extensor digitorum longus muscle excision
The images depict the removal of the EDL muscle. A) Locating the lateral margin of the tibialis anterior (red line). B) Blunt dissection of connective along lateral margin of tibialis anterior, and reflection anteriorly to locate EDL sitting on medial surface. C) Location of the proximal tendon which is then severed and brought down to its distal insertion (black arrow) to be removed.

Figure 6.03 Spinotrapezius muscle excision
These images depict the removal of the spinotrapezius muscles. A) The lateral margin of the spinotrapezius is located at the distal portion of the scapula (black arrow). B) The muscle is then bluntly dissected inferiorly down the spine, removing connective tissue attachments to the spine (black arrow). C) The muscle is dissected down to its inferior insertion at the lumbar region of the spine where it is then removed (black arrow).

Figure 6.04 Sternocleidomastoid muscle excision
The images depict the removal of the sternocleidomastoid muscles of the neck. A) Any excess adipose and thyroid tissue is removed to reveal the sternomastoid and cleidomastoid, laterally (black arrow) and medially (white arrow) respectively. B) The two muscles are bluntly separated before the distal insertion of the two muscles is located at the clavicle (black arrow). C) The muscles insertions are then individually severed and muscles reflected to be removed (white arrow).
6.3 Grimace scale

The above figure represents the grimace scale used post-operatively for determination of rodent pain levels within the first 24hr period. The scale is based on changes in four distinctive areas; orbital tightening, nose/cheek flattening, ear changes and whisker change, all of which are measured on a scale of pain severity (not present, moderate or obvious).